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Effect of pasture biomass intake on growth performance and meat quality of free-range broilers

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ABSTRACT

Effect of pasture biomass intake on growth performance and meat quality of free-range broilers

The effects of forage intake in both broiler performance and poultry meat quality remain to be established. In addition, the potential interest of exogenous cellulases and hemicellulases to improve the nutritive efficacy of free-range chicken remains to be evaluated. Data presented here reveal that pasture consumption promotes bird performance, while contributing for the production of broiler meat with preferred sensory attributes. However, low levels of pasture intake were recorded in birds receiving *ad libitum* a concentrate feed. The restriction of the cereal-based feed intake increased the relative levels of leguminous pasture intake, leading to an overall negative influence in bird performance. In addition, cellulase and hemicellulase supplementation had no impact on the performance of birds foraging in legume-based pastures. The results suggest that in older birds of slow-growing genotypes subjected to free-range production systems, previously unknown sources of beta-glucanases can affect the effectiveness of exogenous enzymes added to the feed. The low levels of pasture intake had a low impact on the fatty acid and vitamin E homologue profiles of free-range poultry meat. When cereal-based feed was restricted, the higher consumption of the leguminous pasture significantly affected meat fatty acid profile. Although pasture intake did not influence the contents in linoleic acid (LA) of poultry meat, the levels of n-3 polyunsaturated fatty acids (PUFA) in breast meat were significantly higher in animals consuming leguminous biomass. Dehydrated forage consumption by conventionally reared broilers had no impact on antioxidant vitamins and cholesterol contents of broiler meats but had a significant effect on meat fatty acid composition. Forage intake did not affect the LA and linolenic acid (ALA) contents in poultry meat, but the levels of n-3 long-chain PUFA in breast meat were higher in animals consuming leguminous biomass. The data suggest an important deposition of ALA in the muscle and the conversion of ALA to its long-chain derivatives in broilers consuming fresh or dehydrated leguminous based forages.

Key-words: free-range broiler; pasture intake; feed enzymes; meat quality; fatty acid composition; recombinant cellulases.

RESUMO

Efeitos da ingestão de pastagem no desempenho produtivo e na qualidade da carne de frangos em crescimento

Os efeitos da ingestão de pastagem no desempenho produtivo e na qualidade da carne de frangos em crescimento, bem como o potencial interesse da suplementação das dietas destes animais com celulases e hemicelulases na produção de frangos ao ar livre continuam por avaliar. Os resultados obtidos neste trabalho revelam que a ingestão de pastagem incrementa o desempenho produtivo e contribui para a melhoria dos atributos sensoriais da carne de frango. No entanto, a ingestão de pastagem foi baixa em aves com acesso *ad libitum* ao alimento composto. A restrição da ingestão do alimento à base de cereais originou um aumento no consumo relativo de pastagem de leguminosas, promovendo o desempenho produtivo das aves. Por outro lado, a suplementação da dieta com celulases e hemicelulases não afectou o crescimento de frangos com acesso a pastagens à base de leguminosas. Os resultados sugerem que em aves de estirpes de crescimento lento criados em sistemas de produção ao ar livre, β-glucanases de origem desconhecida podem afectar a eficiência de enzimas exógenas adicionadas a dietas à base de cevada. A baixa ingestão de pastagem resultou num efeito moderado no perfil de ácidos gordos e homólogos da vitamina E na carne de aves provenientes de sistemas de produção ao ar livre. No entanto, a restrição de alimento composto promoveu uma maior ingestão de pastagem à base de leguminosas, o que influenciou significativamente o perfil de ácidos gordos da carne. Apesar da ingestão de pastagem não influenciar os níveis de ácido linoleico (LA), os teores de n-3 ácidos gordos poli insaturados (PUFA) na carne de frango foram significativamente mais elevados em animais com acesso a pastagem. Igualmente, o consumo de forragem desidratada por frangos produzidos convencionalmente não afectou os teores de vitaminas antioxidantes e de colesterol na carne de frango, tendo influenciado o perfil de ácidos gordos da carne. A ingestão de forragem não afectou os teores de LA e ácido linolénico (ALA). No entanto verificaram-se níveis de n-3 PUFA de cadeia longa de carne de frango mais elevados em animais que consumiram forragem desidratada, o que sugere uma importante deposição de ALA no músculo e a sua conversão nos seus derivados de cadeia longa em aves que consumiram forragens, frescas ou desidratadas, à base de leguminosas.

Palavras-chave: frangos de sistemas extensivos; ingestão de pastagem, suplementação enzimática; celulase recombinante; qualidade da carne; perfil de ácidos gordos.

INTERNATIONAL PEER-REVIEWED PAPERS

This thesis was based on the following publications:

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Percent
+0	Feed not supplemented with a mixture of cellulases and hemicellulases
+E	Feed supplemented with a mixture of cellulases and hemicellulases
18:1ism	18:1 isomers
a*	Redness (CIELAB colour dimension)
ADF	Acid detergent fibre
ADL	Lignin acid detergent
ALA	α-linolenic acid; 18:3 n-3
AME	Apparent metabolizable energy
b*	Yellowness (CIELAB colour dimension)
BW	Body weight
CBM	Carbohydrate binding module
CBM11	Carbohydrate-binding module family 11
cm	Centimetre
Co	Cobalt
***Ct*Lic26A-Cel5E**	*Clostridium thermocellum* Lichenase026A – Cellulase 5E
Cu	Copper
d	Day
DFD	Dry, firm and dark (meat)
DHA	Docosahexaenoic fatty acid; 22:6n-3
DM	Dry matter
-d-old	Day old
DPA	Docosapentaenoic acid; 22:5n-3
E	Enzyme
EPA	Eicosapentaenoic fatty acid; 20:5n-3
FA	Fatty acids
FAME	Fatty acid methyl esters
FCR	feed conversion ratio
Fe	Iron
g	Gram
GH26	β-1,3-1,4-glucanase GH26
GH5	β-1,4-cellulase GH5
GI	Gastrointestinal
h	Hour
HPLC	High-performance liquid chromatography
I	Iodine
IU	International Unit
kDa	KiloDalton
kg	Kilogram
L*	lightness (CIELAB colour dimension)
LA	Linoleic acid; 18:2 n-6
Lab	Free range broilers
LC n-3 PUFA	Long-chain n-3 polyunsaturated fatty acids
m	meters
M	Molar (mol/L)
m^2	Square meter

ME	Metabolizable energy
mg	milligram
min	Minute
MJ	Mega joule
mm	millimetres
mM	Milimolar
Mn	Manganese
MUFA	Monounsaturated fatty acids
n-3	Sum of n-3 Fatty acids = sum of 18:3n-3, 20:3n-3, 20:5n-3, 22:5n-3 and 22:6n-3
n-6	Sum of n-6 = sum of 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6; 22:2n-6 and 22:4n-6
n-6/n-3	n-6/n-3 ratio = sum of 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6; 22:2n-6 and 22:4n-6 / sum of 18:3n-3, 20:3n-3, 20:5n-3, 22:5n-3 and 22:6n-3).
nd	Not determined
NDF	Neutral detergent fibre
NP	No pasture
NSP	Non-starch polysaccharides
ºC	Degree Celsius
P	Pasture
P/S	PUFA/SFA ratio = sum 18:2n-6, 18:3n-6, 18:3n-3, 20:2n-6, 20:3n-6, 20:4n-6, 20:3n-3; 20:5n-3, 22:2n-6, 22:4n-6, 22:5n-3 and 22:6n-3 / sum of 14:0, 15:0, 16:0, 17:0, 18:0 and 20:0
pH	Potential of hydrogen
ppm	Parts per million
PSE	Pale, soft and exudative (meat)
PUFA	Polyunsaturated fatty acids
R	Restriction
Ross	Conventional broilers
S	Season
SA	Soil Association
SCD	Stearoyl-CoA desaturase
Se	Selenium
SEM	Standard error of mean
SFA	Saturated fatty acids
T	Treatment
T. repens	*Trifolium repens*
T. subterraneum	*Trifolium subterraneum*
***Tr*P**	White clover (*Trifolium repens*) based pasture
***Ts*P**	Subterranean clover (*Trifolium subterraneum*) based pasture
U	Units
Zn	Zinc
µg	Microgram

CHAPTER 1 GENERAL INTRODUCTION AND OBJECTIVES

1.1 INTRODUCTION

A growing consciousness of the nutritional importance of human diets for health has led to an increased interest of consumers in specialty poultry products, such as those originated from free-range or organic production systems. Pasture consumption, through outdoor access, is implicit in these alternative production systems, although its importance to broiler performance and poultry meat quality remain to be established. In addition, enzyme supplementation of broilers diets is extensively used in conventional production systems aiming to decrease the anti-nutritive effects of soluble polysaccharides. However, the impact of diet supplementation with fibre degrading biocatalysts on broiler performance in free-range systems remains largely unknown. Globally, this work aims to contribute to improve our current knowledge over both these issues.

In this general introduction, alternative poultry production systems will be summarily reviewed, given special attention to the importance of outdoor access in animal performance. Subsequently, focus will be driven into the use of pasture in poultry diets. Plant cell wall composition and degradation, and the use of enzymes to supplement poultry diets will be approached in the next subchapter. Finally, the influence of the production system on poultry meat quality will be analyzed, given special attention to the lipid fraction of meat. After the analysis of these issues, the specific objectives of this work will be clearly defined.

1.2 ALTERNATIVE POULTRY PRODUCTION SYSTEMS AND OUTDOOR ACCESS

1.2.1 Alternative production systems standards

Alternative poultry production is increasing due to the emergent consumer demand for specialty poultry products from natural or organic systems. Alternative production systems play an important role in attending consumers concerns on health, environment protection

and sustainable agriculture. Alternative production systems, also called extensive production systems, are standardized by specific definitions.

European Union (EU) standards for *Free-Range* and *Traditional Free-Range* (EUC, 1991a) poultry production stipulate maximum indoor and outdoor stocking densities, feed type (at least 70% cereal at finishing diet) and slaughter age. *Free-Range* production systems limit indoor stoking to 13 birds per square meter, and outdoor to at least 1 m^2 per chicken. In addition, birds should be slaughtered with at least 56 days of age. In contrast, *Traditional Free-Range* production standards oblige the use of a slow-growing genotype, limit the number of birds per house, the number of houses on a farm and require larger areas. Stocking density is limited to 12 birds per square meter and outdoor to at least 2 m^2 per chicken. Traditional free-range broilers production systems implicate for a 81 day growing period and a large pop-hole space (4 m length per 100 m^2 of area) to encourage birds to use the outside area. The outdoor area should be covered mainly with vegetation, providing shelter, access to feed and water. Likewise, EU standards for organic production specify stocking density to a maximum of 10 birds per square meter and outdoor to 4 m^2 per chicken as well as the use of feed ingredients that are organically produced (EUC, 1991b). In contrast, United States specialty poultry production classification is relatively vague, with outdoor access required but no specific definitions (USDA, 2007).

1.2.2 Outdoor access

1.2.2.1 The use of outdoor areas by poultry

Alternative production systems for broilers usually involve bird access to an outside area during part of the growing period. Outdoor access allows birds to forage and explore a diverse environment, increasing their choice of environments and food sources, encouraging activity and thus improving bird welfare. Outdoor access provides a large area per bird and decreases animal density, therefore contributing to reduce stress.

The use of outdoor areas by chicken and the possibility of foraging is important for the authenticity of free-range production and consumer confidence (Fanatico, 2006).

However, typically birds reared indoors with outdoor access do not usually access the out door or at best stay within the immediate environment of the houses (Weeks *et al.*, 1994; EUC, 2000).

Broiler exploitation of the outdoor area varies with the season, time of the day, weather conditions, presence of predators and habitat characteristics. Birds make a higher use of outdoor area during the warm summer period, although chicken are less likely to be out at mid-day, both in winter and in bright summer sun, preferring to explore the run area in early morning and in the sun set (Dawkins *et al.*, 2003). Together with full sun, strong wind is the main weather occurrence inhibiting chicken of using the outdoor area (Dawkins *et al.*, 2003; Fanatico, 2006). Overhead predators can restrain poultry of using the outdoor area, which behave adaptively by seeking a cover area (Dawkins *et al.*, 2003; Fanatico, 2006). Attack from above was a danger for their wild ancestors, which is still recognized by the modern poultry breeds (Stahl *et al.*, 2002). In general, poultry prefer ranging areas with trees (Dawkins *et al.*, 2003), which can provide shade from the sun, dry areas for dust bathing, shelter from aerial predators overhead and also a food supply while acting as wind breaks. Taken together these observations suggest that there are positive effects of exposing birds to outside areas.

Some producers move feed and water stations to the outdoors to encourage foraging over a broader area while reducing stoking densities near the house (Fanatico, 2006). Breed also plays a role in foraging intake; with slow-growing genotypes presenting higher levels of forage intake (see section 1.2.4). Another method used by producers to stimulate broilers to graze is to remove their concentrate feed temporarily (Fanatico, 2006). Maintaining doors open late at night and to provide a suitable outdoor habitat, such as providing tree cover (Dawkins et al., 2003), designing pastures specially for poultry (Fanatico, 2006) and integrating poultry production in other livestock production (Hermansen *et al.*, 2004; Glatz *et al.*, 2005; Fanatico, 2006) are all recommended techniques to promote foraging and a more intense use of the outdoor area.

1.2.2.2 Impact of outdoor access on bird performance

Although outdoor access is the main property of alternative production systems, few work have been done to elucidate the impact of outdoor access on bird performance. Production

systems with outdoor access have many factors, such as temperature, photoperiod and light intensity that are not controlled and are naturally variable. Moreover, chicken reared with access to outdoor yards have the possibility to forage, to consume insects and worms that may be available and are allowed to a more intense activity. A lower growth performance is expected in birds with outdoor access, as it was observed in organically reared broilers when compared with birds raised indoors (Castellini *et al.*, 2002b). Although a similar effect on live weight gain was observed in a recent work (Buchanan *et al.*, 2007b), no negative impact was verified on feed efficiency, since the decreased weigh gain resulted from a decreased feed intake. However, data reported by Fanatico and colleagues (2005a) suggest that outdoor access had no impact on weigh gain, feed intake or feed efficiency. Nevertheless, it is clear that the growth performance response to the outdoor access depends on the bird genotype, which will be approached in section 1.2.4.

1.2.3 Housing systems

Housing systems in free-range production systems vary widely, from large fixed houses with yards, to small portable houses that are moved frequently, usually on a day or week base.

Fixed houses are open to yards in order to provide outdoor access and birds are usually closed during nights (see Figure 1.1). Houses are provided with typical equipment and the automation of intensive production systems. This system is often used by large free-range poultry producers. Fenced outdoor area should be covered with vegetation but, the insufficient rotation of birds and the continuous access to the same area, usually results in damage of vegetation and excessive pathogens and nutrient concentration, contributing to disease and pollution. Although *Free Range* standards do not require resting land, different organic production standards recommend a resting period of at least two months a year (EUC, 1991b; SA, 2004).

Figure 1.1 Free-range poultry in fixed houses.
In Panel A, a large flock exploring the outdoor area. In panel B, the evidence of vegetation damage. (Source: http://www.awionline.org)

Portable Houses range from simple shelters to well-constructed and isolated houses (see Figure 1.2). Houses may have wheels or skids and are moved every few days or weekly to a new location. It as been shown that portable houses should be moved at least once a week to prevent that plants underneath the house dye; otherwise the pasture might need to be re-seeded (Fanatico, 2006). Outdoor area should have a perimeter fence to contain other livestock and prevent predators. Electric nets are usually used to define a series of paddocks surrounding the house; allowing birds to be moved thought the paddocks according to the pasture condition.

Figure 1.2 Free-range poultry in portable houses.
(Source: http://www.nfpledbury.co.uk and http://www.farmtek.com)

Pastured Pens are small floorless pens that are moved daily, usually manually, to fresh pasture (see Figure 1.3). This is an inexpensive system preferred by small-scale producers in the United States. The top of the pen is partially covered by roofing. No litter is used so birds can forage. The daily move of the pen allows a better control pathogens related to the continuous contact of birds with is own manure. Since the pen provides the only shelter to the birds, production may be restricted in weather extremes.

Figure 1.3 Free-range poultry in pastured pens.
(Source: http://home.rica.net and http://thechickentractor.com.au)

1.2.4 Poultry genotype, behaviour and welfare

Although most of free range and organic poultry production in United States exploit the fast-growing genotypes used in conventional production systems, the use of slow-growing genotypes is required in European alternative production standards (EUC, 1991a,b), in which the minimum growing period is of at least 81d.

Fast-growing genotypes reared with outdoor access display higher growth performances when compared with slow-growing birds (Castellini *et al.*, 2002a; Gordon & Charles, 2002, Nielsen *et al.*, 2003; Fanatico *et al.*, 2005a). However, fast-growing broilers

advantage vanishes along the rearing period, usually displaying daily weight gains similar to the slow-growing birds. However, equivalent or lower feed conversion ratios are usually observed in slow-growing broilers (Castellini *et al.*, 2002a, Nielsen *et al.*, 2003).

Several studies suggest a better adaptation of slow-growing birds to free-range production systems. Broilers from slow-growing genotypes spent more time in the outdoor area when compared with fast growing birds (Weeks *et al.*, 1994; Castellini, *et al.*, 2002a; Nielsen *et al.*, 2003). In addition, slow-growing birds made a higher use of the outdoor area while fast-growing broilers were mainly found within 4 m of the house (Weeks *et al.*, 1994; Nielsen *et al.*, 2003), which was attributed mainly to leg weakness and lower mobility. These findings reveal a general decrease in activity of fast-growing broilers, at 2 to 3 weeks of age (Pedersen & Thomsen, 2000) due to high growth rates and decreased ability to walk (Bokker & Koene, 2000). Moreover, foraging behaviour differs among genotypes. The analysis of the crop content composition of different strains with outdoor access revealed higher amounts of α-tocopherol and carotenoids in slow-growing birds, indicating higher grass ingestion (Castellini *et al.*, 2002a). Heavy birds have a higher mortality and increased risk of cardiovascular problems and leg weakness (Horn *et al.*, 1998; Jones & Hocking, 1999; Sørensen *et al.*, 2000) and their welfare is consequently more likely compromised. In addition, some authors have reported that selection for rapid growth reduces the immune-competence and increases the susceptibility to environmental disease (Quershi *et al.*, 1998; Rauwn *et al.*, 1998; Yunis *et al.*, 2000).

1.3 PASTURE IN POULTRY DIETS

1.3.1 Pasture as a nutrient source

Usually, the contribution of pasture to bird nutrition is considered insignificant and poultry nutritional requirements are, therefore, usually accomplish exclusively by the concentrate feed. However, in the first half of the 20th century, the majority of poultry were reared using pasture as a primary nutrient source. Pasture was considered an important source of

energy, protein and vitamins which was able to reduce the consumption of concentrated feed by 5% to 10% if birds forage in high quality young pastures (Walker & Gordon, 2003). Considering the nutritional requirements of the actual chicken genotypes, Walker and Gordon (2003) suggested that even if the contribution of grass to the energy, fibre and protein demand were only 5% of the daily demand, it nevertheless represented an important source of nutrients since feed accounts for about 70% of the variable costs of poultry production. Moreover, Fanatico (1998) suggested that maintaining broilers at pasture and allowing birds to forage on plants, seeds, insects and worms, may reduce the levels of concentrates used by 30%.

The extend to which broiler nutritional requirements can be covered by forage consumption can be highly conditioned by the nutritional characteristics of pasture. The nutrient value of pasture depends on a wide range of variables. Plant species and/or varieties sown and the age and maturity of the herbage affect the botanical and chemical composition of pasture. Nutrient value of pasture also changes according to the time of the year and growth stage. Moreover, the botanical composition, and thus the chemical composition, depends also on factors related to farm characteristics and the farmer proceedings such as drainage, compaction, acidity, fertility, weed population, stoking rate and grazing control as well as factors not controlled by the farmer such as altitude, soil type, geology and climate (Pardo & Garcia, 1984; D. G. Crespo, personal communication). Considering the high passage rate in poultry hindgut, a high digestibility of plant biomass is required so that birds can use the forage nutrients more efficiently (D. G. Crespo, personal communication).

Selection of appropriate forage species and adequate pasture management are thus important concerns in the free-range poultry production, although little information is available over these issues. Keeping forage young with high levels of digestibility is one of the key issues of pasture management for poultry (Fanatico, 2006). Young and leafy short plants, under 10 cm long, are preferred by chickens, both for palatability and harvesting ease (Salatin, 1999; Silverman, 2006). In addition, perennial ryegrass based pastures were considered robust alternatives for poultry, while being a good source of energy and fibre with lower levels of protein (Walker & Gordon, 2003). However, pastures displaying a diverse composition are preferred by U.S. producers (Salatin, 1999;

Silverman, 2006). It has been suggested that poultry prefer legumes to grasses (Salatin, 1999). Clover based pastures were shown to display the best results for chicken (Silverman, 2006). Legumes such as clovers display higher proportions of leaf, contributing to a higher digestibility of the animal feed (Karsten, 2003). According to McDonald *et al.* (1989) grass (extensive grazing) has a DM content of 20% and crude protein content of 17.5% on a dry matter basis, whereas white clover (early flowering stage) has a DM content of 19% and crude protein content of 23.7% on a dry matter basis. In an experiment with free-range broilers foraging in two different clover based forages, birds consumed 10.7g DM of herbage per day (Rivera-Ferre *et al.*, 2007). The authors estimated that birds could obtain 0.39g of nitrogen and 62.9 kJ ME (metabolizable energy) per day from the forage, which provided up to respectively 7% and 3% of their daily protein and energy requirements. Additionally, regarding forage digestibility, Buchanan *et al*. (2007a) suggest that poultry have the ability to utilize amino acids found in forage and to obtain small amounts of energy from pasture forages.

1.3.2 Bioactive compounds supplied by forages

Pastures are widely considered to be an important source of a diversity of bioactive compounds, such as polyunsaturated fatty acids (PUFA), pigments, vitamins and saponins, which could have a direct effect on growth performance and on the quality of poultry products.

The lipid content of green forages is variable, ranging from 4 to 12% in a dry matter basis (Morand-Fehr & Tran, 2001), being higher when forages are young and rich in leafs and chloroplasmatic lipids (Hawke, 1973). The fatty acid composition of green forages is characterized by a high percentage of PUFA. The main fatty acid found in leaf lipids is linolenic acid (ALA), which represent 60 to 75% of the total fatty acids, followed by linoleic acid (LA; 18:2 n-6) with 10 to 20% of the total fatty acids (Morand-Fehr & Tran, 2001). Therefore, green forages are also characterized by an n-6/n-3 ratio of about 0.20. Consequently, pasture content in saturated fatty acids (SFA; 10 to 20%) and monounsaturated fatty acids (MUFA; 1 to 17%) is usually low (Morand-Fehr & Tran, 2001). The fatty acid profile in pastures varies with different factors. Young leaves

display higher contents in ALA and are poorer in SFA, when compared with the same plants in a more advanced vegetative state (Morand-Fehr & Tran, 2001). Important differences in fatty acid profile were reported by Gray *et al.* (1967) in the basal region of the leaf when compared with the more mature distal leaf region of the plant, that display higher percentage of ALA and lower content of SFA and oleic acid. The authors suggested that variations on fatty acid composition are related with light exposure, photosynthetic activity and, particularly, chlorophyll content, more than with plant age. As stated previously, pasture management can play an important role in forage fatty acid composition since fatty acid profile of green forages can be conditioned by harvest time (Dewhurst *et al.*, 2001; Clapham *et al.*, 2005).

Although fatty acids represent the major compounds of total lipids in green forages, lipid-soluble pigments and vitamins can reach one third of the plant lipid content (Hudson & Warwick, 1977; Morand-Fehr & Tran, 2001). Chlorophyll is the most important of the plant pigments, playing an essential parte in the process of photosynthesis. Another group of lipid-soluble pigments, associated with chlorophyll, is the yellow carotenoids, which can represent up to 4% of the total lipids of the green plants (Hudson & Warwick, 1977). The main carotenoid present in leaves is β-carotene, which has a recognized role in animal nutrition as a precursor of vitamin A (Britton & Goodwin, 1973). In addition, several oxygenated carotenoids (xanthophylls) are widely found in most plants. Xanthophylls are the carotenoids with most interest in poultry nutrition, considering their effectiveness in influencing yolk and skin colour (NRC, 1994). However, carotenoid levels in plants are affected by several production conditions such as environment, nutrient supply (Britton & Goodwin, 1973) as well as by drying procedures (Sullivan, 1973). Vitamin E, the most important lipid-soluble antioxidant, occurs in eight diterpenes of tocopherols and tocotrienols. α-Tocopherol, the main diterpene found in leaves of grasses and legumes, has an important antioxidant role, inhibiting the peroxidation of unsaturated lipids (Tappel, 1962; Burton & Ingold, 1986).

Additionally, other nutritionally important bioactive factors can be found in plants. Saponins, which are steroid or triterpenoid glycosides, occur in several species of leguminous, such as alfalfa (*Medicago sativa*) and white clover (*Trifolium repens*) (Bondi *et al.*, 1973; Sen *et al.*, 1998). Despite being a well known antinutritional factor, saponins

have been shown to have also beneficial effects such as hypocholesterolemic, anticarcinogenic, anti-inflammatory and antioxidant activity in poultry health (Francis *et al*., 2002; Rao & Gurfinkel, 2000; Ponte *et al.*, 2004c).

1.4 ENZYMES IN POULTRY DIETS

1.4.1 The plant cell wall

1.4.1.1 Structure of the plant cell wall

Carbohydrates, including the low molecular-weight sugars, starch and various cell wall and storage polysaccharides, are de most important energy sources for animals. The plant cell wall is a complex macromolecular structure which is composed by cellulose microfibrils embedded in a matrix of diverse molecules of which the most important are hemicelluloses, pectin and lignin (Cosgrove, 2001).

Many plant cells present two types of cell walls: the primary cell wall (Figure 1.4), which accommodates the cell as it grows, and a secondary cell wall which develops inside the primary wall after the cell has stopped growing. The main chemical component of the primary plant cell wall is cellulose (in the form of organized microfibrils), a complex carbohydrate made up of D-glucose residues. In addition, the cell wall contains two groups of branched polysaccharides, the pectins and hemicelluloses, which chemically and physically are extremely diverse. Hemicelluloses form a complex network of carbohydrates with the cellulose microfibrils, increasing the tensile strength of cellulose, whereas the coextensive network of pectins provides the cell wall with the ability to resist compression.

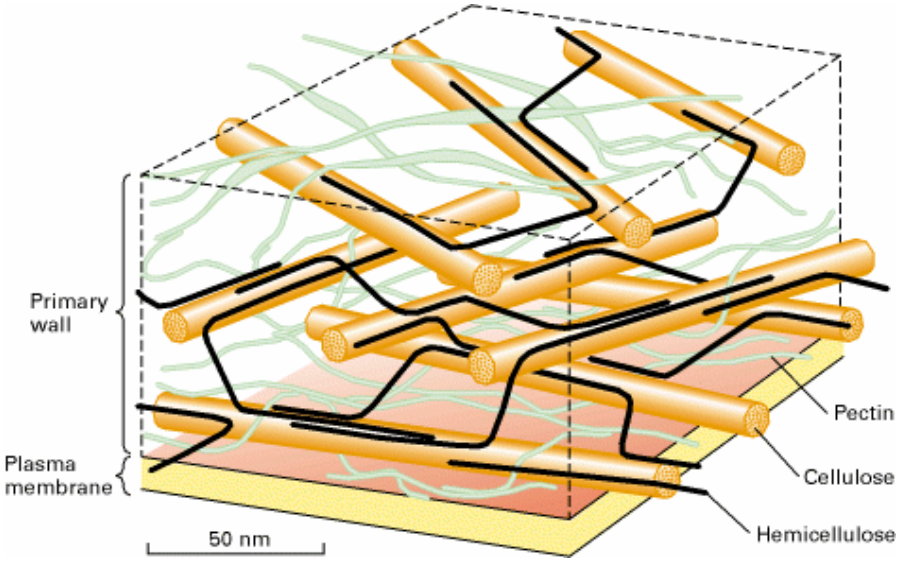

Figure 1.4 Structure of a primary cell wall. The more abundant cellulose fibres are embebed in a matrix of polysaccharides such as hemicellulosesa and pectins. Adapted from Lodish *et al.* (2000).

1.4.1.2 Plant cell wall components

Plant cell wall structural carbohydrates are usually termed non-starch polysaccharides (NSP). NSP are classified in three main groups: cellulose, non-cellulosic polymers (hemicellulose) and pectic polysaccharides. The content of the different components of the plant cell wall vary within plant species, development stage and soil and weather conditions (Heredia *et al.*, 1995).

Cellulose is the main constituent of plant cell walls. It is a linear, insoluble, homopolymer of D-glucose linked by β-1,4-glycosidic bonds. The multiple hydroxyl groups on the glucose residues from one cellulose chain form hydrogen bonds with oxygen molecules on another chain, holding the chains firmly together side-by-side and forming microfibrils with high tensile strength, that confer rigidity to plant cells.

Hemicelluloses (non cellulosic polymers) are the second main component of plant cell walls. Hemicelluloses are heterogeneous polymers of pentoses, hexoses and sugar acids. Unlike cellulose, hemicelluloses are not chemically homogeneous. For example, xylan is a polymer consisting of D-xylose units with β-(1,4) linkages, which can be decorated by a

variety of molecules. Arabinoxylans consist of α-L-arabinofuranose residues attached as branch-points to β-(1,4)-linked D-xylose polymeric backbone chains. The xylose residues may also be linked to other decorating groups such as glucuronic acid residues, ferulic acid crosslinks and acetyl groups (Sørensen *et al.,* 2007). Most of the arabinoxylans in cereal grains are insoluble because they are anchored in the cell walls by alkali-labile ester-like cross links rather than by simple physical entrapment (Mares & Stone, 1973). However, the arabinoxylans not bound to the cell walls are soluble and highly viscous when solubilised (Choct, 1997). β-Glucans consist of a linear chain of glucose units joined by both β-(1,3)- and β-(1,4) linkages (Bengtsson *et al.*, 1990). Although the mixed-linked β-glucans and cellulose are both comprised of β- linked glucose units they are different in their physical properties. Incorporation of the β-(1,3) linkages in the β-glucan molecules breaks the regular structure of β-(1,4) chains, preventing close packaging of the chains while leading to a more soluble polymer. This soluble polysaccharide was shown to form thermo reversible network gels (Fleming & Kawakami, 1977).

Cereals, which are the most used animal feeds, contain significant levels of soluble NSPs such as arabinoxylans and β-glucans. Corn contains very low levels of NSP. In contrast, wheat and rye contain mostly arabinoxylans, whereas barley and oats contain mostly β-glucans. Considering forage crops, such as alfalfa and grasses, it has been shown that when compared with cereals, the levels of soluble NSPs are low, although depending on forage type, stage of growth and season (Barnes *et al.*, 1995).

1.4.1.3 Degradation of plant cell wall polysaccharides

Structural polysaccharides are an important source of carbon and energy in nature. The degradation of this molecules and the use of its by-products are efficiently performed by a wide number of microorganisms, which have developed complex repertoires of enzymes. Considering the remarkable diversity of plant cell wall polysaccharides it is not surprising that the catalysis of these carbohydrates involves the action of a diverse consortium of glycoside hydrolases with different specificities and modes of action. Plant cell wall hydrolysis requires primarily enzymes hydrolyzing β-(1,4) and β-(1,3) glucosidic bonds; β-(1,4) xylosidic bonds and β-(1,4) mannosidic bonds. Plant cell wall hydrolysis cannot be performed by a single microorganism and usually results from the synergistic interaction

of different microorganisms, which act together producing the several enzymes required for the complete degradation of the plant cell wall (Warren, 1996).

In order to characterize the different specificities and mode of actions of plant cell wall hydrolases, the genes encoding enzymes involved in the degradation of the plant cell wall have been cloned and extensively characterized (Tomme *et al.*, 1995). Most of the plant cell wall degrading enzymes display a modular structure comprising one or more catalytic domains linked to secondary domains, often involved in enzyme-substrate targeting that were called CBM (carbohydrate binding module). In addition, non-catalytic domains are also involved in the organization of multienzyme complexes, or cellulosomes, produced by anaerobic cellulolytic microorganisms (Felix & Ljungdahl, 1993; Fontes *et al*., 1995; Tomme *et al.*, 1995).

1.4.2 Anti-nutritive effects of the NSP in poultry

Birds do not possess endogenous enzymes capable of cleaving and degrading plant cell wall polysaccharides. Soluble and insoluble NSP are partially undigested by poultry and are mostly digested at the last portion of the GI tract (Carré *et al.*, 1990). However, soluble NSP are known to display some detrimental effects in poultry nutrition which are associated with the viscous nature of these polysaccharides, their physiological and morphological effects on the digestive tract and the interaction with the gut microflora.

The main detrimental effect reported for insoluble NSP is encapsulation of nutrients (Hesselman & Åman, 1986; Pettersson & Åman, 1989). The insoluble and indigestible polysaccharides of the plant cell wall maintain their integrity through passage in the gastrointestinal tract. Therefore, insoluble NSP act as a physical barrier to digestive enzymes, such as amylase and proteases, reducing the efficient digestion of protein and carbohydrate in the upper part of the gut (Hesselman & Åman, 1986; Choct, 2001).

In contrast, soluble NSP such as arabinoxylans and β-glucans, contribute to increase digesta viscosity (Smits & Anniston, 1996). Hind gut viscosity decreases the rate of diffusion of substrates and digestive enzymes and hinders their effective interaction at the

mucosal surface (Edwards *et al.*, 1988; Ikegami *et al.*, 1990). Increased viscosity caused by soluble NSP is associated with low apparent metabolisable energy (Austin et al., 1999), low nutrient digestibility and absorption (Fengler & Marquardt, 1988; Hesselman & Åman, 1986), and higher incidence of wet and sticky droppings (Smits & Anniston, 1996).

Besides increasing gut viscosity, soluble NSPs also contribute to modify gut functions by altering the endogenous secretion of water, proteins, electrolytes and lipids (Angkanaporn *et al.*, 1994). In general, the consumption of soluble NSP is associated with a range of adaptative changes in the bird's digestive system. The enlargement of digestive organs is described in bird consuming high levels of NSP, as well as an increased secretion of digestive juices and a decreased nutrient digestion. (Pettersson & Åman, 1989; Viveros *et al.*, 1994; Yu *et al.*, 1998). Histological modifications of the jejunum, such as shortening and atrophy of the villi, are also reported in birds feed on high NSP diets (Viveros *et al.*, 1994). Furthermore, certain NSP can bind bile salts, lipids and cholesterol (Vahouny *et al.*, 1981), which can influence the lipid metabolism in the intestine. NSP can also form complexes with digestive enzymes and some regulatory proteins in the gut. Angkanaporn *et al.* (1994) showed that soluble NSP markedly increased endogenous losses of amino acids in chicks. These effects could lead to major changes in the digestive and absorptive dynamics of the gut, with consequent poor overall efficiency in nutrient assimilation by the animal.

The interaction of soluble NSPs with the ecosystem of the gut is referred as a consequence of the increase of the viscosity of the digesta (Angkanaporn *et al.*, 1994; Choct *et al.*, 1996). The slower digesta passage rate in birds consuming NSP rich diets (Salih *et al.*, 1991), with low oxygen available in small intestine, could provide a favourable environment for the anaerobic microflora (Wagner & Thomas, 1978), leading to an increased fermentation in the small intestine (Choct *et al.*, 1996). On the other hand, the slow movement of digesta and the availability of nutrients not used in the small intestine allow for a change of colon and caecum microflora (Annison & Choct, 1991). Since the microflora will also digest and utilize starch and proteins of the digesta, they compete effectively with the host for nutrients (Bedford, 1995). The modification in gut normal microflora can also increase the birds' susceptibility to pathogenic microorganisms, such as *Clostridium perfrigens*, responsible for the necrotic enteritis in poultry (Kaldhusdal &

Hofshagen, 1991; Miles & Jacob, 1997).

Soluble NSPs found in poultry diets directly contribute to impair growth rate, feed conversion efficiency and nutrient digestibility in chickens (Classen *et al.*, 1985; Bedford & Classen, 1992). However, effects of the NSPs are less harmful in adult birds than in young chickens. Several studies suggest that with bird aging, gut microflora adapts to utilize NSPs more efficiently by producing higher levels of digestive enzymes. With age, the gut viscosity of birds fed high NSP diets decreases (Petersen *et al.*, 1999) and NSP digestibility increases (Bolton, 1955; Carré *et al*., 1995).

1.4.3 Enzyme supplementation of NSP rich diets for poultry

Microbial cellulases and hemicellulases are extensively used for supplementing poultry diets rich in NSPs (Bedford, 2000; Fontes *et al.*, 2004). Although a wide number of enzymes are required to perform the complete hydrolysis of plant cell walls, the decrease of the anti-nutritive effects associated with the intake of soluble NSPs can be accomplished by the addition of single endo-acting catalysts (Fontes *et al.*, 2004). In solution, soluble NSPs create viscous solutions by aggregating into large networks. To destroy this network, it is not necessary to digest completely the polymer involved in their constituent monosaccharides, but simply to break the polymers into shorter pieces such that they no longer associate in such large entanglements (Bedford, 1995)

In wheat and rye based diets, rich in arabinoxylans, the addition of xylanase improve weight gain and feed conversion efficiency (Bedford & Classen, 1992; Brenes *et al.*, 1993; Mathlouthi *et al.*, 2002; Meng *et al.*, 2005; Gao *et al.*, 2007). Enzyme addition reduces the intestinal viscosity (Bedford & Classen, 1992; Meng *et al.*, 2005), improve AME of the feed (Mathlouthi *et al.*, 2002; Meng *et al.*, 2005) and increase digestibility of the feed nutrients (Mathlouthi *et al.*, 2002; Gao *et al.*, 2007).

Barley based diets are commonly supplemented with β-glucanase to overcome the antinutritional effects of β-glucans (Hesselman & Åman, 1986; Pettersson *et al.*, 1991). The β-glucans also lead to viscous intestinal contents with all the ensuing problems.

Supplementing broiler diets with exogenous β-glucanase decreases the viscosity of the intestinal content and enhanced weight gain, feed conversion efficiency and nutrient digestibility (Hesselman & Åman, 1986; Brenes *et al.*, 1993; Viveros *et al.*, 1994; Philip *et al.*, 1995). However, the beneficial effect of β-glucanase addition can be highly affected by the content and solubility of β-glucans, which depend on barley strain (Rotter *et al.*, 1989; Nahas & Lefrançois, 2001), heat treatment (Viveros *et al.*, 1994; Vranjes & Wenk, 1995) and preservation method (Svihus *et al.*, 1995; Perttilä *et al.*, 2001).

Although cellulases and hemicellulases are widely used for supplementing cereal-based diets, there is little information available concerning the impact of including plant cell wall hydrolases in diets containing forages. Considering the high NSP content in forages, hydrolases could contribute to a significant depolymerisation of forage plant cell wall polysaccharides resulting in a considerable release of energy, otherwise not available to the animal. In a previous study, exogenous enzymes were not effective in improving the performance of broiler chicks fed on dehydrated alfalfa containing diets (Ponte *et al.*, 2004a). A favourable effect of enzyme supplementation was reported in the performance of birds with access to spring pasture but no similar effect was observed in birds consuming summer forage (Buchanan *et al.*, 2007b). The authors suggest that the capacity of a NSP enzyme to aid the degradation of plant cell wall components is accentuated in early-growth forage in spring months. Finally, considering the possible adaptation of older birds to diet NSPs (see section 1.4.2,), chicken obtain great benefit from enzyme use in their diet when they are juvenile and the advantage of including enzymes becomes smaller as the bird get older (Bedford, 1995; Nahas & Lefrançois, 2001; Olukosi *et al.*, 2007).

1.5 MEAT QUALITY AND SENSORY CHARACTERISTICS OF POULTRY MEAT

1.5.1 Meat physical properties

1.5.1.1 Carcass yield

Within the commercial broiler industry, selection has been successfully used to

increase highly heritable traits such as body weight and breast muscle yield (Havenstein *et al.*, 1994a,b; 2003). The carcass yield is the proportion, usually expressed in percentage, of product recovered from the live bird after processing. The carcass without giblets lies between 64.0% and 68.0% of live weight for broilers and up to 70% for heavier birds (Pollock, 1997). Carcass yield depends mainly on genetics (Havenstein *et al.*, 1994a,b; 2003), with fast-growing genotypes displaying higher carcass yields than slow-growing genotypes (Lewis *et al.*, 1997; Castellini *et al.*, 2002a; Nielsen *et al.*, 2003; Fanatico *et al.*, 2005a). However, environment and production conditions can also influence this parameter. Non optimal temperatures decrease carcass yield (Smith & Teeter, 1987). Low density stocking leads to increased breast yield and decreased tight, drumstick and wing yield (Lewis *et al.*, 1997). Castellini and colleagues (2002b) described increased breast and drumstick percentages when birds had outdoor access and lower stocking density in an organic production system, although outdoor access *per se* did not influence carcass yield (Fanatico *et al.*, 2005a).

1.5.1.2 pH

Postmortem pH decline is one of the most significant events in the conversion of muscle to meat, having a considerable impact on meat texture, colour and water-holding capacity (Fletcher, 1999a; Warriss *et al.* 1999). The rate of pH decline depends on the activity of glycolytic enzymes after death. The ultimate pH is mainly defined by the initial glycogen reserves of the muscle (Bendall, 1973). Low pH is associated with reduced water-holding capacity and reduced functionality, conducing to PSE (pale, soft and exudative) meats (Van Laack *et al.*, 2000; Woelfel *et al.*, 2002). High pH, known as DFD (dry, firm and dark) is associated with poor storage quality due to a faster rate of off-odour production and accelerated microbiological growth (Allen *et al.*, 1997).

Poultry meat pH seems to be controlled genetically (Le Bihan-Duval *et al.*, 1999, 2001). Meat from slow-growing genotypes display lower pH when compared with fast-growing genotypes (Wattanachant *et al.*, 2004; Berry *et al.*, 2005; Debut *et al.*, 2003, 2005; Fanatico *et al.*, 2007a). Intensive selection of broiler chickens, performed to improve growth performances and body composition, leaded to a reduced pH decline of broiler meat (Berri *et al.*, 2001, 2005). The rate and extent of meat pH decline depends also on

environmental pre-slaughter conditions (Holm & Fletcher, 1997). Slow-growing birds are suggested to be more susceptible to stress than fast-growing birds. Active birds from slow-growing genotypes are more vulnerable to shackling stress, which leads to rapid breast muscle acidification. Birds from fast-growing genotypes support better the stressful conditions and meat pH decline is, therefore, slower (Debut *et al.*, 2003, 2005). Production conditions can also influence meat pH drop. Outdoor access induces lower meat pH (Castellini *et al.*, 2002a; Fanatico *et al.*, 2007a). However, this impact is particularly marked in slow-growing strains, which have increased activity (Fanatico *et al.*, 2007a).

1.5.1.3 Colour

Pigmentation is an important factor in consumer acceptance and perceived quality of poultry products (Ouart *et al.*, 1988; Kennedy *et al.*, 2005). Skin and meat colour are influenced by numerous production, handling and processing factors (Fletcher, 1999b). The major contributing factors to poultry meat colour are myoglobin content, the chemical state and reactions of the myoglobin and meat pH (Fletcher, 1999b). Myoglobin content is primarily related to breed, muscle and animal age. As referred above, muscle pH is primarily related to the biochemical state of the muscle at the time of slaughter, which affects both the light reflectance properties of the meat as well as the chemical reactions of the myoglobin. Therefore, muscle pH and meat colour are highly correlated (Le Bihan-Duval *et al.*, 1999, 2001). Higher muscle pH is associated with darker meat whereas lower pH values are associated with lighter meat. Thus, meat colour is mainly influenced by factors affecting meat pH. Genotypes subjected to high selection pressure display decreased colour intensity and increased lightness when compared with less selected genotypes (Le Bihan-Duval *et al.*, 1999; Berri *et al.*, 2001).

Since carcasses are often marketed as a whole, particularly in alternative production markets, skin colour also plays an important role in consumer acceptance. Skin colour is dependent on the genetic capability of the bird to produce melanin pigments in the dermal or epidermal melanophores and the genetic ability to absorb and then deposit carotenoid pigments in the epidermis (Fletcher, 1999b). Carotenoid pigments, which are deposited in the skin, are supplied in the diet. Xanthophylls are the most important carotenoid pigment for poultry pigmentation (Palmer, 1915; Lipstein, 1989). Several studies have been

conducted to evaluate the skin pigmenting properties of a variety of both natural and synthetic sources. Alfalfa and grass meals are widely known as natural sources of xanthophylls for poultry diets (Birckoff *et al.*, 1954; Dansky, 1971; Lipstein, 1989; Ponte *et al.*, 2004a). As referred in section 1.3.2, green pastures can be good suppliers of xanthophylls. Fanatico and colleagues (2007a) referred that birds with outdoor access, which had green forage available for consumption, displayed more deeply pigmented skin. In particular, slow-growing birds exhibited more yellow skin colours, which was attributed to more time spent outdoors, more activity and forage consumption.

1.5.1.4 Texture

Texture is considered the most critical quality factor associated with consumer's ultimate satisfaction of broiler meat products (Fletcher, 2002). Texture is affected by the maturity of the connective tissues and by the contractile state of the myofibrilar proteins. Age at slaughter, genetics and production system are the main factors influencing meat texture.

Collagen cross-linking increases with age and is often associated with increased toughness (Fletcher, 2002). Fast-growing genotypes have larger muscle fibres than slow-growing genotypes, which was associated with reduced proteolytic potential leading to less post-mortem proteolysis and, therefore, reduced tenderization of meat (Dransfield & Sosnicki, 1999). It is generally accepted that breast meat from slow-growing birds is more tender than meat from fast-growing genotypes (Farmer *et al.*, 1997; Fanatico *et al.*, 2007a). However, some studies had described meat from slow-growing genotypes to be less tender compared with meat from fast-growing genotypes (Castellini *et al.*, 2002b; Wattanachant *et al.*, 2004; Fanatico *et al.*, 2005b). However, since slow-growing birds are slaughtered at older ages, an interaction between age and genetics can occur. Nevertheless, the effect of production system on meat texture remains ambiguous. In some studies, outdoor access was shown to result in meat that is more firm than meat generated from indoor production (Castellini *et al.*, 2002c; Santos *et al.*, 2005). Fanatico and colleagues reported that outdoor access resulted in meat that was more tender but only in fast-growing birds (2005b). However, in a recent study Fanatico *et al.* (2007a) reported no impact of the production system on meat texture.

1.5.2 Sensory attributes

The acceptability of meat depends upon qualities such as colour, odour, flavour, juiciness, tenderness, and texture, perceived after cooking. Sensory attributes of poultry meat are influenced mainly by genotype, age, and production system.

Flavour is a combination of the sensations perceived by the two chemical senses, taste and smell. Higher flavour intensity is associated with meat from slow-growing genotypes, which are considered more appropriate for a specialty or gourmet market (Lewis *et al.*, 1997; Castellini *et al.*, 2002c; Gordon & Charles, 2002). Touraille and colleagues (1981) found increases in the intensity of flavour of breast and thigh meat from chickens of slower-growing genotypes. However, the two genotypes under study were compared at different ages and no differences were observed when the genotypes were compared at same age, which leaded the authors to conclude that most of the observed differences were due to an age difference. Older birds are believed to present more intense meat flavours since flavour is intensified after the growth inflection occurs (Farmer, 1999), when flavour precursors are deposited in the muscle (Gordon & Charles, 2002). Alternative production systems, in which birds have outdoor access, can also contribute to meat flavour intensity. Meat from animals that have the opportunity to exercise was suggested to have a more intense flavour (Aberle *et al.*, 2001). According to Gordon and Charles (2002), meat flavour may be modified by access to pasture and the consumption of herbage and live protein. Different forages may result in different flavours and diet manipulation could contribute to change this poultry meat property. However, it is generally accepted that differences in flavour are more associated to animal age at slaughter than with the production system (Farmer *et al.*, 1997; Fanatico *et al.*, 2006, 2007b).

Previous studies (Lawlor *et al.*, 2003; Jahan *et al.*, 2005) that compare the sensory attributes of chicken breast meat from different production systems, which included organic, free range and corn-feed, showed that quality differences determined both by sensory panels and consumers are largely related to appearance and texture (tenderness and toughness), rather than odour and flavour attributes. In a study comparing meat originated in different production systems, conventional meat was considered more tender when compared with alternative meats (Brown *et al.*, 2008). However, the authors

suggested that differences could have been attributed to chicken age and genotype rather to the production system. Older birds are believed to present less tender and firmer meat (Farmer, 1999).

Juiciness is considered important to consumers (Latter-Dubois, 2000). Specialty meat, from slow-growing genotypes with access to the outdoor, was consider less juicy when compared with meat from fast-growing chickens reared indoors (Fanatico *et al.*, 2006, 2007b). The authors suggested that the lower juiciness of breast meat from slow-growing birds may be related to the lower content of intramuscular fat. Breast meat from organic production system had higher scores of juiciness and of overall acceptability (Castellini *et al.*, 2002b). In opposition, Brown *et al.* (2008) described meats from alternative production systems (free range, organic and corn-feed) as being less juicy than meat from conventional birds.

1.5.3 Lipids, cholesterol and fatty acid composition

1.5.3.1 Lipids and human health

Low ratios of polyunsaturated to saturated fatty acids (P/S) in Western diets, as well as an high lipid content, have been considered as major risk factors for cardiovascular diseases, which are among the most important causes of human mortality in developed countries (Katan, 2000; Hu *et al.*, 2001; Ganji *et al.*, 2003). Coronary heart disease and arterioscleroses are highly related to the dietary intake of cholesterol and SFA (Sacks, 2002) and a strong relationship has been demonstrated between cellular cholesterol concentration and Alzheimer's disease (Michikawa, 2003). In addition, PUFA contents of modern diets are low in n-3 fatty acids leading to high n-6/n-3 fatty acid ratios (Simopoulos, 2002).

There is increasing recognition of the health benefits of regular consumption of the long-chain n-3 polyunsaturated fatty acids (LC n-3 PUFA) (FDA, 2004; Kris-Etherton *et al.*, 2003), which extent from development roles, especially in the nervous system, during infancy to the attainment and maintenance of optimal mental and physical health status

throughout adult life (Simopoulos, 1999). In a recent work was demonstrated that although n-6/n-3 PUFA is irrelevant in modifying cardiovascular disease risk, the absolute amounts of dietary LA and ALA are important to the efficiency of conversion of ALA to eicosapentaenoic (EPA; 20:5n-3) and docosahexaenoic (DHA; 22:6n-3) n-3 fatty acids (Griffin, 2008). It has been shown that consumption of EPA and DHA, which are vital components in the retina and the membrane phospholipids of the brain, may reduce the risk of coronary heart disease (Rymer & Givens, 2005) and the incidence of metabolic syndrome (obesity, insulin resistance, or type 2 diabetes and dyslipidemia) (Nugent, 2004). Considering the above discussion, it is widely acknowledged that there is an urgent need to return to a balanced fatty acid diet by decreasing the intake of cholesterol and saturated fats (Evans *et al.*, 2002) and by improving the intake of polyunsaturated fats and n-3 fatty acids (Simopoulos, 2002; Mead *et al.*, 2006).

1.5.3.2 Intramuscular lipid and cholesterol content

Meat and meat products are important sources of fat and cholesterol in human diets. Poultry white meat has recognized low levels of intramuscular fat and cholesterol (Chizzolini *et al.*, 1999; Givens, 2005). Meat fat content depends primarily on genetic characteristics. Breast meat from fast-growing genotypes has a higher lipid content when compared with meats from slow-growing birds (Longeran *et al*., 2003; Havenstein *et al.*, 2003; Fanatico *et al.*, 2007a). In contrast, the effect of the production system on meat lipid content is not clear. Although Castellini *et al.* (2002) described breast meat from organic production system as presenting lower fat content when compared with conventional meat, no differences have been reported when different production systems were compared (Jahan *et al.*, 2004; Fanatico *et al.*, 2005b, 2007a).

In recent years, several feed ingredients have been tested in an attempt to further decease fat and cholesterol content of poultry meat. Dietary garlic and cooper supplements lowered breast muscle and thigh muscle cholesterol (Konjufca *et al.*, 1997), while chia seeds were ineffective in decreasing poultry meat fat and cholesterol content (Ayerza *et al.*, 2002). In a previous work, birds consuming moderate to high levels of dehydrated alfalfa and subjected to high energy feed restriction produced breast meat with significantly lower fat and cholesterol content when compared with birds not consuming

alfalfa (Ponte *et al.*, 2004a).

1.5.3.3 Fatty acid composition

Poultry meat has been considered as one of the main sources of PUFA, in particular n-3 PUFA, for human diets (Sioen *et al.*, 2006; Howe *et al.*, 2006). Although production system and genetics can influence fatty acid profile, diet composition is the main factor modulating fatty acid composition of poultry meat. Meat from birds reared in organic production system, with outdoor access, present higher levels of PUFA, SFA, and n-3 PUFA, particularly LC n-3 PUFA, when compared with birds without access to grass paddock (Castellini *et al.*, 2002b). However, in a study comparing meats available in the market, the organic products had lower contents of certain n-3 PUFA (ALA, EPA and DHA), but higher contents of total PUFA and two n-6 PUFA [LA and arachidonic acid (20:4)] (Jahan *et al.*, 2004). Within the organic production system, meat from slow-growing genotypes displayed a lower SFA content and a higher PUFA and n-3 PUFA, particularly LC n-3 PUFA, when compared with meat from fast-growing birds (Castellini *et al.*, 2006).

The fatty acid profile of non-ruminant meat, such as poultry meat, is essentially a reflection of the FA profile of the diet, since limited transformation of dietary fatty acids occurs during digestion and absorption. Thus, it is theoretically easy to enhance the PUFA and n-3 PUFA content of poultry edible tissues (Hargis & Van Elswyk, 1993; Rymer & Givens, 2005) by dietary manipulation. Several efforts have been made to enhance PUFA and n-3 PUFA contents in poultry meat through the manipulation of the diet composition. Increasing the level of dietary polyunsaturation leaded to an increased accumulation of PUFA in tight and breast meat (Cortinas *et al.*, 2004). Regarding particular fatty acids, increased levels of ALA, LA, EPA and DHA in the diet also leaded to increased contents of these FA in meat (Cortinas *et al.*, 2004). It has been shown, that the content of poultry meat in ALA, can be readily improved by increasing the levels of n-3 PUFA in poultry diets through the incorporation of vegetable oils (López-Ferrer *et al.*, 1999, 2001a). Increasing the levels of EPA and DHA in poultry diets, through the inclusion of oily fish by-products (Hulan *et al.*, 1988; López-Ferrer *et al.*, 2001b) leaded to significant increase of these LC n-3 PUFA. Moreover, marine products are good sources of LC n-3 PUFA,

although their use is restricted due to odour constrains in the final product (Miller & Robish, 1969; Hargis & Van Elswyk, 1993). Therefore the use of LC n-3 PUFA precursors, mainly vegetable oily products, has been studied. ALA may de converted into its long-chain derivatives (Scaife *et al.*, 1994). However, the improvement in the deposition of LC n-3 PUFA is often not nutritionally valuable (Hargis & Van Elswyk, 1993; Scaife *et al.*, 1994; López-Ferrer *et al.*, 1999, 2001b). As referred previously (see section 1.3.2), it is well known that green pastures are a good source of ALA. In ruminants, pasture consumption leads to higher contents of ALA and other PUFA in meat while decreasing the n-6/n-3 fatty acid ratio (Wood & Enser, 1997; O'Sullivan *et al.*, 2004). In birds, Castellini and colleagues (2002) attribute the increased levels of PUFA, particularly EPA and DHA, and total n-3 fatty acids obtained in animals reared in organic production system to the grass consumption.

Chicken meat enriched with PUFA contains longer FA with high number of double bonds, which increases the susceptibility of meat to oxidation (Maraschielo *et al.*, 1999). Lipid oxidation causes loss of nutritional and sensory values as well as the formation of potentially toxic compounds that compromise meat quality and reduce shelf life (Manilla & Husveth, 1999; Bou *et al.*, 2001).

1.5.4 Tocopherols, tocotrienols and β-carotene composition

1.5.4.1 Antioxidants and human health

Although oxidation reactions are crucial for life, they can also be damaging for cells and tissues. Oxidation reactions can produce free radicals, which start chain reactions that damage cells. Free radicals are highly reactive substances damaging cell membranes, injuring heart, vascular, brain, and nervous and muscle systems, and impairing immune competence (Robey & Shermer, 1994). Antioxidants are capable of prohibiting oxidative processes through different mechanisms, thus performing an important role in prevention of disease (Meydani, 2000; Vitaglione *et al.*, 2004; Willcox *et al.*, 2004). Vitamin E is the collective name for a set of eight related tocopherols and tocotrienols, which are fat-soluble vitamins with antioxidant properties (Herrera & Barbas, 2001). Of these, α-

tocopherol is the most important natural antioxidant, as it has the highest bioavailability, being preferentially absorbed and metabolized on this form (Brigélius-Flohé & Traber, 1999). In addition, tocotrienols are known to help lower plasma cholesterol levels (Qureshi *et al*., 1997). β-carotene is a precursor to vitamin A via the action of β-carotene dioxygenase. Besides its antioxidant activity, a recent report demonstrated the effect of β-carotene in the prevention of cognitive decline (Grodstein *et al.*, 2007).

1.5.4.2 Antioxidants and meat quality

PUFA are especially prone to oxidation (see section 1.5.3.3). The increase of the polyunsaturation degree of meat leads to an increased susceptibility to lipid oxidation (Maraschielo *et al.*, 1999; Cortinas *et al.*, 2005). Lipid oxidation can give rise to rancidity and the formation of undesirable odours and flavours, which affects the sensory and the nutritional value of the products. Susceptibility of muscle food to lipid oxidation can be controlled by the presence of antioxidants. Supplementation with α-tocopherol has proven to be an effective way to prevent lipid oxidation (Ruiz *et al.*, 1999; Bou *et al.*, 2001; Grau *et al.*, 2001), improving meat quality, such as colour, flavour, texture, nutritive value and extending shelf life (Morrissey *et al.*, 1994).

It is well known that muscle tissue is easily enriched with α-tocopherol by supplementing diets with this antioxidant (Grau *et al.*, 2001; Bou *et al.*, 2004). However, some authors point out that the protective effect of α-tocopherol against lipid oxidation in chicken meat depends on the lipid profile and the α-tocopherol content of the meat and, hence, of the diet (Maraschiello *et al.*, 1999; Ruiz *et al.*, 1999; Grau *et al.*, 2001). Accordingly, the content of oxidable substrate, such as PUFA, in diet and tissues is important for the achievable enrichment level (Frigg *et al.*, 1991). The increase of dietary contents of PUFA leads to a decreased deposition of α-tocopherol in tight muscle (Cortinas *et al.*, 2003), which was explained by the use of α-tocopherol for the prevention of oxidative processes in tissue in vivo, since diets with low PUFA contents resulted in a significant enrichment of α-tocopherol in thigh meat. Additionally, positive effects of α-tocopherol were reported on functional properties of chicken meat, mainly under heat stress conditions and α-tocopherol supplementation (Olivo *et al.*, 2001). Considering the antioxidant capacity of β-carotene, the effectiveness of this substance is not as conclusive as for α-tocopherol,

since its effects depend on the dose and on the type of fat fed (King *et al.*, 1995; Ruiz *et al.*, 1999).

As referred above, pastures are good sources of tocopherols, tocotrienols, and β-carotene. In ruminants, the consumption of grass with naturally high vitamin E content leads to the deposition of this antioxidant in muscle and fat so that oxidation is retarded and meat shelf-life is enhanced (Wood & Enser, 1997). In poultry, data concerning the improvement of meat oxidative stability of birds consuming pasture is scarce. Castellini *et al.* (2002b) obtained a lower oxidative stability in organically reared chicken, with access to a grass paddock, which was attributed to the greater degree of physical activity and the consequent increase in muscle oxidative capacity (Petersen *et al.*, 1997). Within the organic production system, meat from slow-growing birds display lower oxidative stability than meat from fast-growing birds, although higher amounts of α-tocopherol and β-carotene were found in the crop content of slow-growing birds suggesting an environmental higher antioxidant capacity (Castellini *et al.*, 2006). The greater locomotion activity and the uncontrolled environmental conditions were pointed out by the authors as the cause of the increased oxidability (Castellini *et al.*, 2006).

1.6 OBJECTIVES

In this study, we aimed to elucidate several unsolved questions concerning the production of free-range poultry in leguminous-based pasture. Particularly, the aspects of pasture consumption in bird performance and meat quality were evaluated.

The specific objectives of this work were:

- To study the effect of pasture intake and enzyme supplementation on performance, meat quality and sensory attributes of pastured poultry (Chapter 2).
- To investigate the effect of pasture consumption on the fatty acid composition, cholesterol, tocopherol and tocotrienol contents of meat from pastured broiler chicken (Chapter 2).

- To evaluate the capacity of a bifunctional recombinant derivative of *Ct*Lic26A-Cel5E from *Clostridium thermocellum*, to enhance nutritive value of a barley based diet for free-range pastured birds of a slow-growing genotype (Chapter 3).

- To study the effect of restricting the intake of a cereal-based feed in free range pastured poultry on performance, meat quality, and meat fatty acid profile, cholesterol, and lipid-soluble antioxidant vitamins (vitamin E homologues and β-carotene) contents of the meat (Chapter 4).

- To establish the impact of including a dehydrated leguminous-based forage in broiler diets on bird performance and on meat fatty acid profile, and cholesterol, and lipid-soluble antioxidant vitamins (vitamin E homologues and β-carotene) contents of animals of a fast-growing genotype exploited under an intensive production system (Chapter 5).

CHAPTER 2 EFFECT OF PASTURE INTAKE AND ENZYME SUPLEMENTATION ON PERFORMANCE AND MEAT QUALITY OF FREE-RANGE BROILERS

2.1 PASTURE INTAKE IMPROVES THE PERFORMANCE AND MEAT SENSORY ATTRIBUTES OF FREE-RANGE BROILERS

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ABSTRACT

Free-range chickens are assumed to consume low to moderate levels of pasture, although the effects of forage intake in both broiler performance and poultry meat quality remain to be established. In addition, in spite of cellulases and hemicellulases being widely used as feed supplements to improve the nutritive value of cereal-based diets for fast growing broilers, it remains to be established the potential interest of these biocatalysts in the production of free-range chicken. In this study broilers of the RedBro Cou Nu × RedBro M genotype were fed on a cereal-based diet in portable floorless pens located either on a rainfed subterranean clover (*Trifolium subterraneum*) pasture or on an irrigated white clover (*Trifolium repens*) pasture. Control birds were maintained at the same site in identical pens but with no access to pastures. The importance of pasture intake and enzyme supplementation in the performance and meat sensory properties of the free-range chicken, from day 28 to 56, was investigated. The results revealed that while cellulase and hemicellulase supplementation had no impact in broiler performance ($P>0.05$), birds foraging in legume-based pastures reached significantly higher final body weights. The data suggests that the improvement in broiler performance results from an increase in the intake of the cereal-based feed rather than from an improvement in the efficiency of nutrient utilization per se. Interestingly, although the intake of the subterraneum clover pasture had no impact on the tenderness, juiciness and taste of broiler meat, members of a 30-person consumer panel classified the meat from grazing broilers with the higher scores in a global appreciation. Together the results suggest that pasture intake promotes bird performance while contributing for the production of broiler meat with preferred sensory attributes.

Key words: free-range broiler, pasture intake, broiler performance, meat quality

2.1.1 Introduction

In recent years consumer interest in specialty poultry products derived from free-range or organic production systems has been steadily increasing in both the United States and in Europe (Fanatico *et al.*, 2006). Consumer preference for poultry speciality products is related with a perceived higher quality and security of meat derived from such systems coupled with high standards of animal welfare, although in most cases there is little scientific evidence supporting these options (Latter-Dubois, 2000). Under these systems, animals have access to an outside area promoting foraging, feed selection and activity and thus, theoretically, improving the welfare of the birds. While outdoor access is intrinsic to the free-range system, there are large variations concerning the amount and type of outdoor access provided in most of the free-range and organic systems that are presently in practice in Europe and the U.S. Therefore, although outside access is associated with pasture and invertebrate consumption the nutritional value derived from the intake of such products is presently unknown and will obviously vary dramatically with the system in use (Walker & Gordon, 2003).

In the United States, small farmers have adopted a free-range poultry production method that promotes pasture intake, which was termed the pastured poultry system (http://www.apppa.org). At three or four weeks of age broilers are introduced into floorless portable pens that are moved daily to fresh pasture in order to encourage forage intake. Compared with conventional free-range and organic systems, the pastured poultry alternative is likely to induce considerable higher levels of pasture consumption and, therefore, it is an ideal system to evaluate the nutritional impact of pasture intake in broiler performance and meat quality. Pasture may constitute a source of energy and protein for growing broilers. In addition, the presence of a large range of bioactive compounds in the forage, such as xanthophylls and several hypocholesterolemic and anticarcinogenic compounds, may lead to improvements in meat quality (Ponte *et al.*, 2004c). However, the high fibre content of pasture biomass may limit nutrient utilization and could reduce growth rates and feed efficiency. To our knowledge, the effects of pasture intake in broiler performance and meat quality in free-range systems remain largely unknown.

Microbial cellulases and hemicellulases are widely used for supplementing poultry diets

rich in NSPs (Bedford, 2000; Fontes *et al.*, 2004). Soluble arabinoxylans and β-glucans lead to a considerable increase in digesta viscosity, therefore interfering with the movement of particles and solutes across the intestinal lumen and reducing the access of the repertoire of digestive enzymes to their substrates (Bedford *et al.*, 1991; Edwards *et al.*, 1988). Endo-acting polysaccharide hydrolases added to the diets decrease the degree of polymerization of the recalcitrant NSPs, leading to a considerable reduction in digesta viscosities (Bedford & Classen, 1992). In addition, breakdown of plant cell wall polysaccharides improves the access of the digestive biocatalysts to the endosperm contents that were otherwise trapped (Chesson, 1993). However, it is still unknown if cellulases and hemicellulases could contribute to improve the nutrient utilization of pasture biomass from free-range broilers. For this application polysaccharidases would have to contribute to a significant hydrolysis of the recalcitrant carbohydrates at the upper part of the GI tract so that more energy could be absorbed in both the small intestine and in the hind gut.

The objective of this study was to establish the impact of pasture intake on broiler performance and resulting meat quality. Free-range broilers were allowed access to subterranean clover (*T. subterraneum*) or white clover (*T. repens*) based pastures. Birds of a control group remained in the same site but without access to the pastures, in order to allow a rigorous identification of the effects of herbage intake. The capacity of cellulases and hemicellulases to improve the nutritive value of diets containing significant percentages of forage was investigated. In addition, the sensory attributes of meat derived from the described production systems were evaluated. Finally, a comprehensive characterization of the fatty acid profile and the contents in cholesterol, tocopherols and tocotrienols of broiler meat derived from such treatments is presented in the accompanying paper (Ponte *et al.*, 2008b; section 2.2).

2.1.2 Material and methods

2.1.2.1 Animals, diets and management

Two experiments (Spring and Autumn) were conducted in the spring and autumn of 2003 at Herdade dos Esquerdos (039º 07.18' north, 007º 29.36' west, 318 m a.s.l.), Vaiamonte, Portugal, using the same trial design, to determine the effect of pasture intake and cellulase and hemicellulase supplementation on broiler performance and meat sensory characteristics. In the Spring Experiment, the average daily mean temperature was 13.7 ºC (mean of highest temperatures 20.0 ºC and of the minimum 7.3 ºC), with 86.4 mm of rain occurring during six days. In the Autumn Experiment, the average daily mean temperature was 12.3 ºC (mean of highest temperatures 17.8 ºC and of the minimum 6.9 ºC) with nine days with rain and a total precipitation of 128.7 mm. For each experiment, two hundred and forty 28-d-old males RedBro Cou Nu x RedBro M, vaccinated against Marek disease, were divided into 24 floorless portable metal outdoor pens (10 birds per pen/replicate), equalizing both the mean and the variance of body weight (BW). Animals were maintained in the pastured pens described below for an additional 28 days till slaughtered at d 56. The movable pens allowed birds to directly contact the legume-based pastures (see Figure 2.1), promoting forage intake, and measured 1.7 m × 1.5 m × 0.5 m (0.255 m^2 per bird). Approximately one third of the top of each cage area was covered with transparent white-washed plastic for protection against harsh climatic conditions. In general, the pens were very effective in protecting birds from predators although ground predators (foxes) were able to invade a few pens during the experiments. Water and a cereal-based feed were available *ad libitum* throughout the experiments and were provided in two automatic drinking nipples and in an individual hanging tube feeder, respectively. The composition of the basal diet used on these studies, which was formulated to contain adequate nutrient levels as defined by the NRC (1994), is presented in Table 2.1.

Figure 2.1 Aspect of the movable pens for pastured poultry used in these studies.

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

Ingredients	**%**
Corn	44.1
Wheat	26.0
Soybean meal 47%	27.0
Salt	0.30
Calcium carbonate	0.78
Dicalcium phosphate	0.98
Choline 75%	0.06
DL-Methionine	0.18
Mineral and vitamin premix[1]	0.60
Calculated nutrient content	
Energy (MJ ME/kg DM)	12.1
Crude Protein (%)	19.1
Ether extract (%)	2.50
Crude fibre (%)	5.00
Ash (%)	6.00
Methionine (%)	0.42

[1]Mineral-vitamin premix provided the following per kilogram of diet: vitamin A, 9,000 IU; vitamin D3, 2,100 IU; vitamin E, 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, 100mg; Zn, 80 mg; Co, 0.2 mg; I, 1.0 mg; Se, 0.3 mg; monensin, 100 ppm.

The birds were randomly assigned into one of the six treatments with 4 replicates of ten birds per treatment. The six treatments consisted of two levels of enzyme supplementation, with (+E) or without (+0) a cellulase and hemicellulases enzyme mixture, and three types of pasture consisting of a irrigated white clover (*Trifolium repens*) pasture (*Tr*P), a subterranean clover (*Trifolium subterraneum*) pasture (*Ts*P) and no pasture (NP), in a completely randomized experiment. At day 42, half-way through the experiments, samples of both pastures were collected from 1 m^2 paddocks, by cutting it at 3 cm above the ground, for proximate analysis that was performed as described below. The chemical composition of the pasture samples is presented in Table 2.2. The cellulase and hemicellulase supplement was added to the cereal-based diet at a level of 0,41 % (w/w) and consisted of a mixture of 1:10:30 of Roxazyme G (Roche Vitamins Ltd., Basel, Switzerland), Avizyme 1300 (Danisco Animal Nutrition, Wiltshire, UK) and Avizyme 1100 (Danisco Animal Nutrition, Wiltshire, UK). Roxazyme G, contains a minimum of 1600 U/g of cellulase, 3600 U/g of endo-1,3(4)-β-glucanase and 5200 U/g of endo-1,4-β-xylanase, while Avizyme 1300 contains a minimum of 300 U/g of endo-1,4-β-xylanase, 300 U/g of endo-1,3(4)-β-glucanase and 800 U/g protease and Avizyme 1100 contains a minimum of 2500 U/g of endo-1,4-β-xylanase and 800 U/g protease. While the Roxazyme G supplement was incorporated at the recommended level, the incorporation levels of Avizyme 1100 and 1300 were 4 and 1.3 times higher, respectively, of the recommended doses. This enzyme mixture was used in an attempt to hydrolyze at least part of the cellulose and the hemicelluloses fractions of the pasture. To promote forage intake, the portable pens of the treatments with access to pasture were moved daily so that birds could dispose of fresh herbage every day. The two pastures used by the birds in these experiments were contiguous, in order to avoid climate variations, and were installed in the autumn of 2002. The White clover pasture was irrigated during the dry summer season (June-September). Pens of the no pasture treatment were located in a fixed position, in the same field, and access to the pasture was blocked (see Figure 2.2), in the initial days and throughout the experiment, by adding new pine wood shavings to the ground.

Figure 2.2 Pens in which birds had no access to the pasture. To avoid grazing outside the pen hay was used to cover the surrounding of each facility.

Table 2.2 Chemical composition of the legume-based pastures used by the free-range broilers in the spring and autumn experiments (% DM).

	Spring		**Autumn**	
	T. subterraneum	***T. repens***	***T. subterraneum***	***T. repens***
Dry matter	16.67	15.20	12.47	14.57
Crude protein	20.78	26.78	30.97	23.02
Ether extract	2.66	2.05	3.01	3.80
NDF	46.90	38.32	34.57	35.29
ADF	24.59	22.87	24.21	29.34
ADL	4.49	4.43	3.89	12.14
Hemicellulose	22.30	15.45	10.36	5.95
Cellulose	20.11	18.44	20.32	17.2

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 56, one bird per cage was slaughtered by an intravenous injection of an aqueous solution of 125 mg Tiopental Brown and digesta was collected

from the various gastrointestinal compartments. Levels of cellulase and hemicellulase activity in the GI tract were measured as described below. The dry matter weight of forage and cereal-based feed found in the crop was measured, allowing the estimation of pasture consumption considering the levels of cereal-based feed ingested. In addition, at the end of the experiments, at day 56, six birds per pen were slaughtered at a commercial processing plant. At the same day, twenty four 35-d-old Ross 308 commercial broilers (here termed Ross), raised under the conventional system, and twenty four 81-d-old RedBro Cou Nu x RedBro M broilers raised under the EU free-range system (here termed Lab) were slaughtered at the same commercial processing plant. The Ross birds were randomly selected from four different farms (six birds per farm), while Lab birds were also selected from birds originated from four different farms (six birds per farm). The carcasses were refrigerated for 24 hours and weighed. Meat pH was measured as described by Sierra (1973). Carcasses were frozen at -20ºC for later sensory and texture evaluation.

2.1.2.2 Analytical procedures

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). Cellulase and xylanase assays were performed using carboxymethylcellulose and oat spelt xylan, respectively, according to the methods described by Fontes *et al.* (2000). Analysis of cellulase and xylanase activity in the digesta contents recovered from the various gastrointestinal compartments was assessed in agar plates, using the polysaccharides referred above at 0.1% (w/v) final concentration, in 10 mM Tris HCl pH 7.0. Activity was detected after 16 hours incubation at 37 ºC through the Congo Red assay plate, as described in Ponte *et al.* (2004b) and Mourão *et al.* (2006).

2.1.2.3 Microbial evaluation

Prevalence of Campylobacter and Salmonella species on farm was determined by monitoring the presence of both bacteria in animal faeces, water and the cereal-based feed. At the beginning and at the end of both experiments (animals with 28 and 56 days of age, respectively) samples of water and of the basal feed (25 g) were collected for microbial quantification (n=5). In addition, on the same periods twenty faecal samples were

randomly collected from animals of the 6 treatments by cloacal swab using sterile cotton-tipped swabs. Campylobacter and Salmonella were detected and quantified following the methods described by Musgrove *et al.* (2001) and McCrea *et al.* (2006), respectively, which essentially follow the International standard methods ISO/FDIS 10272-1 (2005) and ISO 6579 (2002).

2.1.2.4 Skin colour

The colour of breast skin was evaluated using a Minolta chroma meter CR-300. The readings were taken on equivalent positions of the carcasses. The tip of the chromameter-measuring head was placed flat against the surface of the skin. For each reading 3 measurements were performed and the final value for each animal is the average of those readings. Skin colour was expressed in the CIELAB dimensions of lightness (L*), redness (a*) and yellowness (b*). Skin colour evaluation was performed before the carcasses were frozen at -20 ºC.

2.1.2.5 Sample preparation for sensory and shear force analysis

Approximately four weeks after slaughtering, a consumer test was conducted on the breast meat at Estação Zootécnica Nacional kitchen/sensory facility. Carcasses were thawed at refrigerated temperature (4 ºC) and cooked for 40 min in a standard commercial oven at 200 ºC, such that the final internal temperature of the meat was 65ºC (± 5ºC). From each carcass, half of the breast was used for sensory evaluation and the other half was prepared for shear force values by cutting two 1.9-cm-wide strips. Only *pectoralis major* muscle was used for shearing force evaluation using a Warner-Bratzler shear device, attached to a TA-tx2i Texture Analyser (Stable Micro Systems). The measurements of maximum shear force were taken on equivalent positions of the strip. Triplicate shear measurements were recorded on each breast and averaged.

2.1.2.6 Sensory analysis

The sensory evaluation of meat samples from the spring experiment was performed by a sensory panel that was not screened for behaviour such as poultry consumption habits or free-range poultry purchasing. The sensory panel consisted of 30 untrained consumers, chosen from the staff of Estação Zootécnica Nacional, who had previously participated

in similar sensory evaluations. Panel members were not given any information about the meat or the experimental treatments and procedures. Serving sizes were half a split breast piece served without the skin. Panellists were asked to evaluate liking of tenderness, juiciness, flavour and global appreciation of each meat sample individually, in a 1-5 scale (1- very disagreeable; 2- disagreeable; 3- neither agreeable nor disagreeable; 4- agreeable; 5- very agreeable).

2.1.2.7 Statistical analysis

Statistical analysis was conducted by analysis of variance using SAS with the GLM procedure (SAS, 2004). The experimental unit considered was the pen. In relation to the animal performance data, initially the model considered the effects of pasture intake, season, enzyme supplementation and the interactions between the various effects. Since none of the interactions was found significant ($P>0.84$), they were removed from the model. Unless otherwise stated, differences were considered significant when $P<0.05$.

2.1.3 Results and discussion

The contribution of foraging to the nutrition of free-range chicken remains largely unknown. To evaluate the importance of pasture intake and enzyme supplementation in the performance and meat sensory attributes of free-range broilers, two experiments were conducted in the spring and autumn with broiler chicks foraging on legume-based pastures. In order to effectively access the importance of forage intake, control birds were kept in the same field, exposed to the same experimental conditions, although foraging on the legume-based pastures was not allowed. Therefore, nutrient supply in the control birds (NP) derived exclusively from the cereal-based feed, which was available, for all groups, *ad libitum*. In addition, a heavy load of cellulases and hemicellulases was used to investigate the capacity of polysaccharidase supplementation to improve the nutritive value of the forage for broiler chicks. The mortality rates during the two experiments were 9% (spring) and 16% (autumn) and were mainly related (60% of the cases) with fox invasion of the pens (results not shown).

Although the nutritive value of the herbage changes according to the time of the year, both pastures displayed relatively high crude protein contents, in both the spring and autumn, as a consequence of the predominance of leguminous species (Figure 2.3; Table 2.2**).** However, DM percentages were always relatively low (see Table 2.2**)** and it is clear that NDF remains the main organic component of the pasture. Although chickens have been reported to feed on a wide range of macro-invertebrates living in the surface soil (Clark & Gage, 1996), their contribution to the diet of free-range broilers was not quantified in these experiments.

Figure 2.3 Leguminous-based pastures

2.1.3.1 Bird performance

The results of the two experiments, expressed as final body weight, weight gain, feed intake and feed conversion ratios are summarized in Table 2.3. In both seasons, the final body weights of birds consuming pasture were significantly higher than that of the control animals kept under the same environmental conditions and, therefore, exposed to identical temperature, precipitation and photoperiod fluctuations, but that were not allowed foraging. The differences in the final body weights are related with the higher weight gains of grazing birds, which ranged from 75 to 150 g more of body weight when compared with the non-grazing animals in the four weeks that the experiment lasted. The data suggest that, in general, pasture intake promoted an increase in the consumption of the cereal-based feed. In the spring experiment, consumption of the cereal-based feed

showed a trend for increase in animals in the *T. repens* pasture (P=0,107) and this trend is also manifested for the birds of the subterranean clover pasture, although the differences relative to the control birds were not significant (Table 2.3)**.** Interestingly, in the autumn experiment animals consuming forage had always higher intakes of the cereal-based feed when compared with the non-grazing birds, although the intakes were even higher for the broilers in the subterranean clover pasture. These data suggest that differences on the levels of cereal-based feed consumption may be related with the composition and/or the levels of pasture intake as it will be discussed below. There were no differences between the feed conversion ratios of animals subjected to the three different grazing regimes, suggesting that bird performance primarily depends on the intake of the cereal-based feed rather than from an improvement on the efficiency of nutrient utilization per se. Finally, it is interesting to verify that, considering the theoretical suboptimal environmental conditions to which the free- range chicken were subjected when compared with birds housed indoors, the growth rate achieved by the broilers, in both the spring and the autumn experiment, is at the levels expected for the genotype RedBro Cou Nu x RedBro M (2079 g of BW at day 56 – Hubbard ISA management manual). However, feed conversion ratios were considerably higher than expected for this genotype (should be 2.1-2.2 at day 56), suggesting that animals can compensate growth at inappropriate temperatures, humidity and light intensity by increasing feed intakes. The difference between our results for feed conversion and the ones referred by the Hubbard ISA management manual can also result from different energetic concentrations of diets: the cereal-based feed had 12.12 MJ EMA/kg instead 13.38 MJ EMA/kg as recommended in the management manual.

Table 2.3 Performance of free-range broilers fed on a cereal-based feed supplemented (+E) or not supplemented (+0) with a mixture of cellulases and hemicellulases, without access to pasture (NP) or foraging in *Trifolium subterraneum* (*Ts*P) or *T. repens* (*Tr*P) based pastures.

	Final Body Weight[1] (g)	**Weight Gain (g)**	**Feed Intake[2] (g)**	**Feed Conversion[2] Ratio**
Spring Experiment				
NP	1950.3 [a]	1231.0 [a]	3443.8[a*]	2.817
*Ts*P	2069.1 [b]	1349.6 [b]	3518.9[ab*]	2.695
*Tr*P	2117.3 [b]	1390.6 [b]	3665.9[b*]	2.707
SEM	43.45	40.51	87.35	0.0891
+0	2032.6	1309.6	3566.0	2.797
+E	2058.4	1338.0	3519.7	2.681
SEM	28.23	26.32	56.76	0.0579
Autumn Experiment				
NP	2106.7 [a]	1258.5 [a]	3550.8[a]	2.979
*Ts*P	2211.9 [b]	1367.2 [b]	4046.4[c]	3.087
*Tr*P	2178.5 [b]	1334.8 [b]	3837.0[b]	2.928
SEM	24.49	23.54	66.17	0.0852
+0	2182.3	1336.7	3839.2	3.006
+E	2149.2	1303.7	3783.5	2.990
SEM	20.52	19.72	55.44	0.0711

[1] Initial body weights were of 719 and 840 g for the spring and autumn experiments, respectively. [2] Feed intake and feed conversion are relative to the cereal-based feed. [a,b] Means with the same column bearing different superscripts are significantly different ($P<0.05$ or $P=0.107$ when *).

Attempts made in the spring experiment to estimate forage intake by the free-range birds, based on the evaluation of the levels of biomass present in the pastures before and after grazing, failed as a result of the heterogeneous nature of the pastures and the low grazing levels of birds. Therefore, a second method based on the quantification of the proportion of pasture and the cereal-based feed found in the crops of sacrificed birds at the end of the experiment, was implemented in the autumn experiment. It was found that grass biomass represented between 2.5-4.5 % on a DM basis or 18-26 % on a fresh basis, of the total feed intake in the grazing animals. Although these values should be viewed with some caution, since they represent an estimate of the pasture consumption at a specific moment of the trial and forage consumption may have varied during the 28 days of the experiment and even during the same day, they represent a crude first estimate of biomass intake in

free-range broilers.

The capacity of a complex mixture of cellulases and hemicellulases, consisting of a large array of plant cell wall hydrolytic activities, to improve the nutritive value of diets of pasture broilers was evaluated. The data presented in Table 2.3 suggests that supplementation of the cereal-based feed with heavy doses of exogenous polysaccharidases was unable to significantly improve bird performance in both the spring and the autumn experiment. It is possible, that the inability of polysaccharidases to improve the performance of free-range chicken results from enzyme inhibition and/or proteolysis in the animals GI tract. To discard this possibility, digesta samples were collected from the various gastrointestinal compartments and tested for cellulase and xylanase activity. The data (not shown) demonstrated that high levels of both cellulase and xylanase activities were present in the crop, duodenum and jejunum of animals fed with the cereal-based feed supplemented with the plant cell wall hydrolases. Under the same conditions, no enzyme was detected on the corresponding compartments of animals fed on the basal diet without exogenous enzymes. As expected, all animals, supplemented or not with the microbial enzymes, display high levels of polysaccharidase activity in the ceacum (not shown). Together, the data suggest that the incapacity of enzymes to improve animal performance may result from the low intake of pasture material (2.5-4.5 % on a DM basis). In addition, the complexity of pasture plant cell wall polysaccharides may require higher enzyme doses eventually with different enzyme specificities acting for longer periods than that allowed from the short digestive transit period of chicken.

2.1.3.2 On farm microbial contamination

Campylobacter and Salmonella are the two leading sources of food-borne illness in Europe and the United States. The prevalence of infected chicken is usually higher in free-range birds when compared with animals in enclosed housing, since outdoor birds putatively have an increased exposure to additional vectors of infection (McCrea *et al.*, 2006). Therefore, during the field experiments the prevalence of Campylobacter and Salmonella on farm was determined. No positive samples were encountered at both the beginning and at the end of the experiments, in the water, cereal-based feed and animals (data not shown), suggesting that birds were neither contaminated at the commencement

of the experiments nor got infected in the 28 d outdoor trial. These data are unusual considering the considerable prevalence of both bacteria on free-range chicken farms as reported by other studies (Heuer *et al.*, 2001, Rivoal *et al.*, 1999, McCrea *et al.*, 2006). While the number of samples analyzed in each experiment might have been low (thirty at the beginning and thirty at the end), it is possible that the on site conditions were particularly favourable to avoid microbial contamination since pastures were previously never used by other chickens on grazing experiments. Although the potential for the transmission of food safety pathogens to humans through free-range poultry products is real, it is clear that the prevalence of the pathogens can be low and will vary widely with the on farm conditions.

2.1.3.3 Meat physical properties

The influence of the production system, particularly pasture intake, in the various aspects of the overall quality of poultry meat was investigated. In the accompanying paper (Ponte *et al.*, 2008b; section 2.2) aspects concerning the biochemical properties of broiler chicken meat derived from these experiments will be described. In the present study the influence of pasture intake in carcass yield, meat pH and texture, and skin colour were evaluated. Since enzyme supplementation had no influence in broiler performance, experiments with meat samples were performed exclusively with meat samples of animals not supplemented with the exogenous enzymes. The data, presented inTable 2.4**,** showed that pasture intake had a positive effect on carcass yield in both experiments. This is unexpected since the expected higher activity of grazing animals was believed to improve the proportion of wings, thighs and drum sticks, while foraging could increase the proportion of GI tract tissues on the overall BW. However, Fanatico *et al.* (2005a) found no differences in the carcass yield of indoor and outdoor birds. Therefore, it is possible that carcass yield may have been affected by two factors: birds with pasture had a more developed gastrointestinal tract (due to a higher fibre intake and total feed intake) that reduce carcass yield but had a higher body weight that create a trend to increase it.

Table 2.4 Carcass yield and breast meat shear force and pH of free-range broilers fed on a cereal-based feed without access to pasture (NP) or foraging in *Trifolium subterraneum* (*Ts*P) or *T. repens* (*Tr*P) based pastures.
Meat physical parameters of conventional grown broilers slaughtered at day 35 (Ross) or free range broilers of the RedBro Cou Nu x RedBro M genotype slaughtered at day 81 (Lab) were determined for comparison.

Treatment	Carcass yield (%)	Breast meat pH	Shear force (kg)
Spring Experiment			
NP	65.4 [a]	5.70 [a†]	3226.7 [a]
*Ts*P	66.5 [b]	5.80 [b†]	3111.3 [a]
*Tr*P	67.1 [b]	5.81 [b†]	3318.1 [a]
Lab	nd	5.72 [a†]	3710.6 [b]
Ross	nd	5.78 [b†]	2930.0 [a]
SEM	3.90	0.017	146.65
Autumn Experiment			
NP	64.9 [a]	5.87 [a†]	2962.6
*Ts*P	67.7 [b]	5.86 [a†]	3495.8
*Tr*P	66.8 [b]	5.81 [a†]	nd
Lab	nd	5.90 [a†]	3797.8
Ross	nd	6.09 [b†]	3555.1
SEM	5.90	0.033	339.08

nd, not determined. [a,b] Means with the same column bearing different superscripts are significantly different ($P<0.01$ or $P<0.0001$ when [†]).

Breast meat pH was higher in meats originated from grazing animals in the spring experiments (Table 2.4). This is surprising since several studies have indicated a decrease in the pH of meats from outdoor reared pigs and chicken, reflecting better welfare conditions, reduced pre-slaughter stress and thus reduced consumption of glycogen (Enfält *et al.*, 1997; Castellini *et al.*, 2002b). Interestingly, meat from the older animals displayed consistently higher pH values. Tenderness is an important attribute for consumers and was measured by evaluating meat shear force values. Overall, the data demonstrated that pasture intake did not affect meat texture (Table 2.4), such that, in the spring experiment, NP meat had a similar (P>0.05) shear force to that observed in the *Ts*P and *Tr*P groups, while in the Autumn experiment, shear force was similar (P>0.10) in the four groups tested. However, conventional free-range chickens with 81 days of age were shown to

have produced less tender meat, an observation that might be directly correlated with a higher proportion of cross-linked collagen in older birds.

Results of the colorimetric evaluation of breast skin are presented as the CIELAB values of L (lightness), a (redness) and b (yellowness) in Table 2.5. In general, pasture intake did not influence broiler skin colour. However, in the spring experiment, birds foraging on the *T. repens* pasture displayed higher L scores, indicating a less deeply pigmented skin. Interestingly, in both experiments animals from the non pasture treatment displayed a considerable increase in the broiler carcasses redness (a), showing that the usually undesirable pink and red tones in the skin were more developed. Overall, the data suggests the skin from NP, *Ts*P and *Tr*P birds had higher b values, compared to the commercial broilers, suggesting a higher efficacy of the cereal-based feed for pigmenting the carcasses with yellow tones, which may result from its high proportion in corn. This is supported by the observation that although pasture contains carotenoid pigments (Toyopmizu *et al.*, 2001), no improvement of the yellowness of the breast skin colour was observed when diets contain a considerable proportion of corn (Schaible, 1970). In addition, the increased levels of cereal based feed ingested by animals foraging on the clover based pastures had no influence on the carcass yellowness, suggesting that pigments supplied by the corn based feed were already present at a saturating level in non-foraging birds.

Table 2.5 Breast skin colour of free-range broilers fed on a cereal-based feed without access to pasture (NP) or foraging in *Trifolium subterraneum* (*Ts*P) or *T. repens* (*Tr*P) based pastures.
Breast skin colour of conventional grown broilers slaughtered at day 35 (Ross) or free range broilers of the RedBro Cou Nu x RedBro M genotype slaughtered at day 81 (Lab) was determined for comparison.

Treatment	L* (lightness)	a* (redness)	b* (yellowness)
Spring Experiment			
NP	57.69 [b]	0.698 [c]	12.85 [b]
*Ts*P	58.55 [b]	0.235 [bc]	12.71 [b]
*Tr*P	59.98 [c]	0.145 [b]	12.89 [b]
Lab	55.36 [a]	-1.059 [a]	9.42 [a]
Ross	60.04 [c]	-0.718 [a]	10.93 [a]
SEM	0.448	0.2018	0.525
Autumn Experiment			
NP	59.57	1.160 [c]	13.74 [b]
*Ts*P	58.89	0.418 [bc]	13.00 [b]
*Tr*P	58.53	0.431 [bc]	11.57 [ab]
Lab	58.33	-0.495 [a]	14.21 [b]
Ross	60.96	-0.339 [b]	9.43 [a]
SEM	0.704	0.2886	1.081

[a,b] Means with the same column bearing different superscripts are significantly different ($P<0.05$).

2.1.3.4 Sensory evaluation

Sensory evaluation of the breast meat focused on tenderness, juiciness, taste and overall acceptance. The sensory experiments were exclusively performed with meat of birds from subterraneum clover pastures, to allow the comparison with commercial meats without affecting the robustness of the statistical analysis. The data, presented in Table 2.6, suggests that the intake of subterranean clover pasture had no effect in meat tenderness, juiciness and taste. In addition, the panel was unable to discriminate meat originated from commercial and NP and *Ts*P birds in terms of juiciness and taste. As expected, meat originated from conventional free-range chicken, slaughtered at day 81, was classified as less tender when compared with meat of birds from the fast growing genotype (Ross), slaughtered at day 35, or meat from the NP and *Ts*P birds of this study (slaughtered at day

56). In contrast, the younger age and fast growing genotype of the Ross should have contributed to the classification of the meat as more tender. Differences in tenderness may be due to the fact that fast growth in birds leads to larger muscle fibres and differences in proteolytic potential (Dransfield & Sosnicki, 1999). However, it is possible that in some conditions differences in texture are subtle and not differentiated by the consumers.

Table 2.6 Impact of pasture intake on sensory attributes of chicken breast meat.
Experimental meat was obtained from free-range broilers fed on a cereal-based feed without access to pasture (NP) or foraging in *Trifolium subterraneum* (*Ts*P) based pastures. Sensory parameters of meat from conventional grown broilers slaughtered at day 35 (Ross) or free range broilers of the RedBro Cou Nu x RedBro M genotype slaughtered at day 81 (Lab) were determined for comparison.

	Tenderness	**Juiciness**	**Taste**	**Overall Appreciation**
NP	3.73 [b]	3.23	3.40	3.33 [ab]
*Ts*P	3.97 [b]	3.30	3.77	3.77 [c]
Ross	3.80 [b]	3.27	3.47	3.56 [bc]
Lab	3.30 [a]	3.00	3.37	3.10 [a]
SEM	0.120	0.162	0.145	0.146

Grading scale: 1- very disagreeable; 2- disagreeable; 3- neither agreeable neither nor disagreeable; 4- agreeable; 5- very agreeable.
[a,b] Means with the same column bearing different superscripts are significantly different ($P<0.01$).

Although tenderness is usually suggested to be the most important organoleptic attribute of meat (Seabra *et al.*, 2001), in a general appreciation consumers classified meat from birds that graze the subterranean clover pasture with the highest values. Although it is possible that different leguminous species may result in different flavours and therefore different sensory attributes (Gordon and Charles, 2002), due to the analytical composition similarity of subterranean clover and white clover it is unlikely that white clover pastures would lead to a different general appreciation of the meat. Therefore, these data provide a good indication that pasture intake, even when consumed at reduced levels, generates broiler meat with higher degrees of consumer acceptability. Although not quantified, birds from the pasture experiments were observed to spend more time in activity and therefore it could be argued that the different sensory attributes result from the broiler increased activity. However, recent work suggests that sensory properties of chicken meat do not depend on the increased activity of the broiler (Fanatico *et al.*, 2006). Interestingly,

the overall acceptance of meat from conventional free-range chicken was not different from meats of *Ts*P or NP birds, although the former birds were slaughtered at an older age (81 days compared with 56 of the pasture experimental chicken). Older birds are believed to present less tender and firmer meat and more intense meat flavours since flavour increases after the growth inflection (Farmer, 1999). However, no differences on meat tenderness, juiciness and taste were perceived between meats of 81 and 56 day old birds. Nevertheless, conventional free-range chickens have access to grass in the outdoors, although levels of pasture intake are usually low but variable since birds forage in the proximity of the buildings. Therefore, an overall acceptance value of 3.56 for the conventional free-range meat (between the 3.33 and 3.77 values of NP and *Ts*P meats, respectively), may reflect low intakes of pasture of the commercial birds. Surprisingly, although the meat from the conventional fast growing chicken was classified as the most tender by the panel, its overall acceptance had the lowest scores, although not different from NP birds. Together the data suggests that the slow growing genotype produces meat with higher sensory attributes, when compared with the fast growing Ross genotype, while pasture intake can further improve its intrinsic overall acceptance.

In conclusion, in this study the supplementation of a cereal-based diet for pastured broilers with a heavy load of microbial cellulases and hemicellulases had no impact on broiler performance. In contrast, the data suggests that pasture intake promotes growth by improving the consumption of the cereal-based feed, although the levels of forage intake (on a DM basis) were low. Together the data presented here and in the accompanying study (Ponte *et al.*, 2008b; section 2.2), suggest that pasture intake improves meat sensory attributes, supporting the consumer assumption that poultry products derived from free-range pastured-based systems present higher standards of sensory quality.

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2.2 INFLUENCE OF PASTURE INTAKE ON THE FATTY ACID COMPOSITION, CHOLESTEROL, TOCOPHEROLS AND TOCOTRIENOLS IN MEAT FROM FREE-RANGE BROILERS

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ABSTRACT

Over the last centuries, Western diets acquired a dramatic imbalance in the ratio of P/S with the concomitant reduction on the dietary proportion of n-3 PUFA. Pastures are a good source of n-3 fatty acids, although the effect of forage intake in the fatty acid profile of meat from free range chicken remains to be evaluated. In addition, it is unknown if consumer interest in specialty poultry products derived from free-range or organic production systems is accompanied by an higher nutritional quality of these products. In this study broilers of the RedBro Cou Nu × RedBro M genotype were fed on a cereal-based diet in portable floorless pens located either on subterranean clover (*Trifolium subterraneum)* or white clover (*Trifolium repens)* pastures. Control birds were maintained at the same site in identical pens but had no access to pastures. The capacity of ingested forage to modulate broiler meat fatty acid profiles and the meat content in total cholesterol, tocopherols and tocotrienols was investigated in broiler chicks slaughtered at day 56. The results suggested that pasture intake (<5% DM) had a low impact on the fatty acid and vitamin E homologue profiles of meat from free-range broilers. However, breast meat from animals with free access to pasture presented lower levels of the n-6 and n-3 fatty acid precursors, LA (18:2n-6) and ALA (18:3n-3), respectively. In addition, in spring the levels of EPA (20:5n-3) in breast meat were significantly higher in animals consuming pastures, which suggest a higher conversion of ALA into EPA in these birds. Finally, when compared with meat from slower growing genotypes obtained under the conventional European free-range production systems with slaughtering at day 81, meat from birds of the Ross genotype raised intensively and slaughtered at day 35, seems to present an higher nutritional quality.

***Key words*:** free-range broiler, pasture intake, fatty acid profile, P/S

2.2.1 Introduction

Low ratios of polyunsaturated to saturated fatty acids (P/S) in Western diets have been considered as major risk factors for cardiovascular diseases, which are among the most important causes of human mortality in developed countries (Hu *et al*., 2001; Ganji *et al*., 2003). In addition, PUFA contents of modern diets are low in n-3 fatty acids leading to high n-6/n-3 fatty acid ratios (Simopoulos, 2002). The imbalance in the n-6 *versus* n-3 proportion is responsible for the pathogenesis of many diseases, including cardiovascular disease, cancer, and inflammatory and autoimmune diseases (Simopoulos, 2004). In addition, it has been shown that consumption of EPA and DHA n-3 fatty acids, which are vital components in the retina and the membrane phospholipids of the brain, may reduce the risk of coronary heart disease (Rymer & Givens, 2005). Considering the above discussion, it is widely acknowledged that there is an urgent need to return to a balanced fatty acid diet by improving the intake of polyunsaturated fats and n-3 fatty acids (Simopoulos, 2002).

Poultry meat has been considered as one of the main sources of PUFA, in particular n-3 PUFA, for human diets (Sioen *et al*., 2006; Howe *et al*., 2006). It has been shown, that the content of poultry meat in n-3 fatty acids, particularly in ALA, can be readily improved by increasing the levels of n-3 PUFA in poultry diets through the incorporation of vegetable oils (López-Ferrer *et al*., 1999, 2001a) and/or oily fish by-products (Hulan *et al*., 1988; López-Ferrer *et al*., 2001b). However, a decrease in flavour quality has been reported for these products due to an overall higher meat susceptibility to lipid oxidation (Manilla & Husveth, 1999; Bou *et al*., 2001). It is well known that green pastures are a good source of ALA and pasture consumption leads, in ruminants, to higher contents of this fatty acid in meat while decreasing the n-6/n-3 fatty acid ratio (Wood & Enser, 1997; O'Sullivan *et al*., 2004). Free-range chickens are expected to consume variable levels of forages, although pasture contribution to alter meat fatty acid profiles in chicken remains to be evaluated. In addition, although pasture is a poor source of EPA and DHA, it is presently unknown if birds have the capacity of using pasture ALA as a precursor for the synthesis and deposition of these two essential fatty acids in broiler meat. Moreover, pastures are a good source of tocopherols and tocotrienols, the natural diterpenes with vitamin E activity, which is the primary lipid-soluble antioxidant in biological systems (Kerry *et*

al., 2000). Tocotrienols are also known to help lower plasma cholesterol levels (Qureshi *et al*., 1997). Antioxidant supplementation of feed is an efficient method for increasing meat oxidative stability (Maraschiello *et al*., 1999), although the various vitamin E forms are known to present different antioxidant potencies (Bourgeois, 1992). The contribution of grass vitamin E homologues for the oxidative stability of meat from free-range chicken remains, however, to be established. Finally, meat provides from one third to one half of the daily-recommended cholesterol intake (300 mg, WHO), which seems to be directly associated to a greater risk of hypercholesterolemia (Chizzolini *et al.*, 1999). Nevertheless, the influence of pasture intake in cholesterol levels in free-range chicken remains unknown.

Consumer interest for organic and natural poultry products is increasing in Western societies. In Europe, the free-range broiler production systems use slow-growing meat birds with a production period of at least 81 d (European Union Commission Regulation, 1991; Fanatico *et al*., 2005a). The birds are housed in conventional production plans but the animals are allowed free access to the outdoor during the day and are fed *ad libitum* with diets containing more than 70% of cereals. Pasture consumption under this system is likely to be low since animals tend to rapidly spoil the small surplus of grass found in the vicinity of the buildings. Not only the production system but also the bird's genotype, size and age may affect meat fatty acid profiles (Rymer & Givens, 2005). Therefore, research is needed in order to evaluate the impact of the above mentioned factors in the fatty acid profile of meat from free-range broilers when compared with meat from conventional grown birds.

In order to access the impact of pasture intake in bird performance and meat quality, floorless portable pens were used to produce broilers of a slow-growing genotype from days 28-56 in legume-based pastures. Data presented in the accompanying paper show that pasture intake promotes boiler performance while not affecting meat sensorial attributes (Ponte *et al*., 2008a; section 2.1). The objective of the research reported here was to investigate the effect of pasture consumption on the fatty acid composition, cholesterol and vitamin E compounds of meat from free-range chicken. In addition, the cholesterol content and the profiles of fatty acids and vitamin E homologues in meat of broilers originated on free-range and conventional production systems will be

characterized.

2.2.2 Material and methods

2.2.2.1 Reagents

Analytical grade and liquid chromatographic grade chemicals were from Merck Biosciences (Darmstadt, Germany). Sodium methoxide (0.5 M solution in anhydrous methanol) was from Sigma-Aldrich (St. Louis, MO, USA) and fatty acid methyl esters (FAME) standards were from NU-Chek-Prep. Inc. (Elysian, MN, USA) and Supelco Inc. (Bellefonte, PA, USA). Absolute ethanol (99.8%) was purchased from AGA (Lisbon, Portugal). *n*-Hexane, isopropanol (Merck Biosciences, Darmstadt, Germany) and Milli Q water were of HPLC-grade. High-purity nitrogen gas (R grade) was acquired from Air Liquide (Lisbon, Portugal). Tocopherols and tocotrienols standards were obtained from Calbiochem (Merck Biosciences, Darmstadt, Germany), and cholesterol and β-carotene standards from Sigma Chemical Co. (St. Louis, MO, USA).

2.2.2.2 Animals, diets and management

Two experiments were conducted in the spring and autumn of 2003 in Herdade dos Esquerdos (039º 07.18' north, 007º 29.36' west, 318 m), using the same trial design. In the spring experiment, the average of the daily mean temperature was 13.7 ºC (mean of highest temperatures 20.0 ºC and of the minimum 7.3 ºC) with 6 days with rain and a total precipitation of 86.4 mm. In the autumn trial, the average of the daily mean temperature was 12.3 ºC (mean of highest temperatures 17.8 ºC and of the minimum 6.9 ºC) with 9 days with rain and a total precipitation of 128.7 mm. For each experiment, one hundred and twenty 28-d-old males RedBro Cou Nu × RedBro M, vaccinated against Marek disease, were divided into 12 floorless portable outdoor pens (10 birds per pen/replicate), equalizing both the mean and the variance of body weight (BW). Before the commencement of the experiment, from days 0 to 28, animals were raised in battery brooders in a temperature-controlled room under standard brooding practices and were fed *ad libitum* with a typical maize-soybean diet. At day 28, animals were moved to the

pastured pens described below, in which they were maintained for an additional 28 days till slaughtered at d 56. The portable pens measured 1.7 m × 1.5 m × 0.5 m (0.255 m^2 per bird) and allowed birds to directly contact the legume-based pastures, promoting pasture intake. Water and a cereal-based feed were available *ad libitum* throughout the experiments and were provided in two automatic drinking nipples and in an individual hanging tube feeder, respectively. The composition of the cereal-based feed used on these studies, which was formulated to contain adequate nutrient levels as defined by the NRC (1994), is presented in Table 2.1**.** Approximately one third of the top area of the pen was covered with a transparent white-washed plastic to protect against harsh climatic conditions.

For both experiments, the birds were randomly assigned into one of the 3 treatments with 4 replicates of 10 birds per treatment. The 3 treatments consisted on birds fed *ad libitum* with a cereal-based feed without access to pasture (NP), with access to a white clover (*Trifolium* repens) based pasture (*Tr*P) or with access to a subterraneum clover (*T. subterraneum*) based pasture (*Ts*P). At day 42, half-way through the experiments, samples of both pastures were collected from 1 m^2 paddocks, by cutting it at 3 cm above the ground, for chemical analysis. During these experiments, pasture biomass was shown to contain between 12-17 % of dry matter, 20-31 % of crude protein and 15-19 % of crude fibre, being the last values expressed on a dry matter basis (see section 2.1). To promote pasture intake, the portable pens of the treatments with access to pasture were moved daily so that birds could eat pasture every day. The 2 pastures used by the birds in these experiments were contiguous, in order to avoid climate variations, and were installed in the autumn of 2002. The white clover based pasture was irrigated during the dry summer season (June-September). Pens of the no pasture treatment were located in a fixed position, in the same field, and access to the pasture was blocked, in the initial days and throughout the experiments, by adding new pine wood shavings to the ground. At the end of the experiments, at day 56, 6 birds per pen were slaughtered at a commercial processing plant (24 birds per treatment). In addition, at day 56, one bird per cage was slaughtered by an intravenous injection of an aqueous solution of 125 mg Tiopental Brown and the proportion of forage and cereal-based feed found in the crop was measured to estimate pasture consumption.

At slaughter day of birds from the spring experiment, twenty four carcasses of 35 day-old Ross commercial broilers (meat of this treatment was referred as Ross), raised under the conventional system and slaughtered at the same commercial processing plant, were acquired for further comparison with the field experimental meats. In addition, twenty four carcasses of 81 day-old RedBro Cou Nu × RedBro M, raised under the EU free-range system (meat of this treatment was referred as Lab) were obtained, to allow comparing the biochemical properties of the Ross and Lab meats. The Ross and Lab birds were randomly selected from birds originated from four different conventional or free-range farms, respectively, with a total of six birds slaughtered per farm. Animals that originated meats of the Ross and Lab treatments were fed *ad libitum* with typical maize soybean diets, although the precise composition of the feeds is unknown. The Ross and Lab treatments aim to represent poultry meats available for consumers in the market. Carcasses were stored in a cool chamber at 0 to 4 ºC for 24 h. After carcass measurements, skinless breast meat samples (approximately 10 g) were collected for determining total lipids, fatty acid composition, total cholesterol and vitamin E compounds, ground using a food processor (3 × 5 s), vacuum packed and stored at -80 ºC until required.

2.2.2.3 Determination of total lipids

Meat samples were lyophilized (-60 ºC and 2.0 hPa) to constant weight using a lyophilisator Edwards Modulyo (Edwads High Vaccum International, UK), maintained desiccated at room temperature and analyzed within two weeks. For total lipid determination, intramuscular fat was extracted as described by Alfaia *et al*. (2006) from the lyophilized samples (0.25 g). Total lipids were measured gravimetrically, in duplicate, by weighing the fatty residue obtained after solvent evaporation.

2.2.2.4 Determination of fatty acid composition

Intramuscular fat of lyophilized samples (0.25 g), cereal-based feed or pasture (0.10 g of dry matter) were firstly dissolved in 1 ml of dry toluene. Then, fatty acids were converted to methyl esters (FAME) by base-catalyzed transesterification with sodium methoxide for 2 h at 30 ºC. The fatty acid composition was determined by gas chromatography of FAME, performed with a gas chromatograph Varian 3800 (Varian Inc, Walnut Creek, CA, USA) equipped with a flame ionization detector and an OmegaWax 250 (Supelco,

Bellefont, CA, USA) capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness). The chromatographic conditions were as follows: injector temperature, 250 ºC; detector temperature, 280 ºC; helium was used as carrier gas and the split ratio was 1:20. The gas chromatograph oven temperature was programmed to start at 150 ºC (maintained for 15 min) followed by a 3 ºC/min ramp to 220 ºC (maintained for 20 min). Identification was accomplished by comparing the retention times of peaks from samples with those of FAME standard mixtures. 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 %, using the corrected response factor of each fatty acid (ES ISO 5508, 1990). Fatty acids were expressed as gravimetric contents (mg g^{-1} muscle) or as a percentage of the sum of identified fatty acids (% w/w).

2.2.2.5 Quantification of total cholesterol, tocopherols and tocotrienols

The simultaneous determination of total cholesterol, β-carotene, tocopherols and tocotrienols was performed as described by Prates *et al*. (2006). The method involves a direct saponification of the fresh meat (0.75 g), high-energy feed or pasture (0.10 g of dry matter), a single *n*-hexane extraction and the analysis of the extracted compounds by normal-phase HPLC, using fluorescence (tocopherols and tocotrienols) and UV-Vis photodiode array (cholesterol and β-carotene) detections in tandem. The contents of total cholesterol, β-carotene, 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.

2.2.2.6 Statistical analysis

Statistical analysis was conducted by analysis of variance using SAS with the GLM procedure (SAS Institute, 2004). The model used for analysing data of the pasture experiment included the effect of treatment (T), the effect of season (S) and the interaction between T and S (T×S). The experimental unit considered was the pen. In Table 2.8 and Table 2.9 and Table 2.10, the significance for main effects of season and interaction were presented but the treatment effect was replaced by two orthogonal contrasts. The first contrast (NP *vs.* P) compared the biochemical parameters of meat from birds with no

access to pasture (NP) with meat from animals that had access to pasture (P). The second contrast (*Tr vs. Ts*) compared the biochemical parameters of meat from birds with access to the white clover based pasture (*Tr*) with meat from animals with access to the subterraneum clover based pasture (*Ts*). The linear model used in the experiment of commercial broilers included only the effect of the type of commercial production (Lab and Ross). In this specific case, the experimental unit considered was the farm. Unless otherwise stated, differences were considered significant when $P<0.05$.

2.2.3 Results and discussion

The contribution of foraging to change meat fatty acids, cholesterol, tocopherols and tocotrienols in free-range chicken remains largely unknown. In a series of two experiments, in the spring and autumn of 2003, the effect of pasture intake in the performance of broiler free-range chicken was evaluated. Data presented in the accompanying paper (Ponte *et al*., 2008a; section 2.1), revealed that grass biomass represent between 2.5-4.5 %, on a DM basis (18-26 % on a fresh basis), of the total feed intake in grazing animals. In addition, pasture consumption promotes higher intakes of a cereal-based diet available *ad libitum*, leading to increased body weights in animals with access to the legume-based pastures. Interestingly, meat from free-range broilers grazing in subterraneum clover based pastures had differentiable and preferred sensory properties. Here, meat samples from animals of the described experiment were used to evaluate the effect of incorporating subterraneum clover or white clover in the diets of free-range broilers in meat fat and vitamin E composition.

2.2.3.1 Fatty acid composition, cholesterol, β-Carotene, tocopherols and tocotrienols of pastures and cereal-based feed

The fatty acid composition of the cereal-based feed and of both pastures is presented in Table 2.7. Total fatty acids were higher in the cereal-based feed, intermediate in pastures from autumn and lower in pastures from spring. As expected, LA was the major fatty acid in the cereal-based diet, while ALA predominates in the legume-based pastures, especially in the autumn. Palmitic acid (16:0) is relatively abundant in the various feeds, although

with higher levels in the pastures relative to cereal-based feed. In contrast, the cereal-based feed contained higher percentages of oleic acid (18:1n-9) when compared with all pastures. There were no major differences in the fatty acid profile of the subterraneum clover or white clover based pastures, although in the autumn both pastures had a higher proportion of ALA. In addition, EPA and DHA were vestigial in all the feeds analyzed (data not shown). Finally, the four pastures presented ratios of LA/ALA below 0.40, while the cereal-based feed depicted an LA/ALA ratio of 16.7.

Table 2.7 Total fatty acids (mg g^{-1} dry matter), fatty acid composition (% w/w), diterpenes (tocopherols and tocotrienols) and β-carotene (μg g^{-1} dry matter) of the cereal-based feed and of the *Trifolium repens* (*Tr*P) and *T. subterraneum* (*Ts*P) based pastures used in the autumn and spring experiments.

	Cereal-based feed[1]	**Autumn**		**Spring**	
		***Tr*P**	***Ts*P**	***Tr*P**	***Ts*P**
Total fatty acids	21.4	12.3	12.9	6.25	5.04
Fatty acids					
14:0	0.08	1.00	0.73	1.04	1.20
16:0	14.0	20.4	23.5	32.3	32.3
16:1n-7	0.19	0.42	0.39	0.00	0.00
17:0	0.15	0.41	0.22	0.71	0.41
18:0	2.63	3.28	2.85	5.26	7.46
18:1n-9	21.5	3.09	2.78	6.10	5.50
18:2n-6	57.6	12.2	8.38	15.1	12.3
18:3n-3	3.45	58.4	60.5	37.9	39.3
20:0	0.37	0.65	0.62	1.58	1.50
Diterpenes					
α-Tocopherol	36.9	25.3	42.9	27.9	15.5
α-Tocotrienol	6.48	19.1	13.9	8.82	8.96
β-Tocopherol	2.17	1.41	1.49	1.02	0.85
γ-Tocopherol[2]	20.5	6.12	13.3	2.77	2.09
γ-Tocotrienol	3.60	50.3	27.9	27.3	22.7
δ-Tocopherol	1.49	0.66	1.00	0.53	0.92
δ-Tocotrienol	nd	7.71	4.48	2.30	2.86
β-Carotene	nd	10.8	21.4	3.13	2.96

[1] The cereal-based feed was supplemented with α-tocopherol (20 mg kg^{-1}); [2] Co-eluted with a small proportion of β-tocotrienol and, in the cereal-based feed, with δ-tocotrienol; nd, not detected.

The diterpenes (tocopherols and tocotrienols) content of the feedstuffs used in these experiments are also shown in Table 2.7. Although γ-tocopherol was co-eluted with a minor proportion of β-tocotrienol and, specifically in the cereal-based feed, with δ-tocotrienol a complete profile of vitamin E compounds was obtained. The α- and γ-tocopherols are the most abundant vitamin E homologues in the cereal-based diet, in accordance with the exogenous supplementation of α-tocopherol to the cereal-based feed, whereas α-tocopherol and γ-tocotrienol predominate in the legume-based pastures. It is well known that tocotrienols have different antioxidant potencies and biological activities when compared with tocopherols and, therefore, the determination of all vitamin E molecules in feed is critical. In addition, the pastures presented significant levels of β-carotene, although the cereal-based feed showed undetectable levels of this lipid-soluble antioxidant pro-vitamin.

2.2.3.2 Effect of pasture intake on fatty acid composition, cholesterol, tocopherols and tocotrienols of meat from free-range broilers

Data referring to the fatty acid composition of breast meat from free-range broilers fed *ad libitum* on a cereal-based diet and allowed grazing in subterraneum clover or white clover based pastures, during spring and autumn, are presented in Table 2.8 and in Table 2.9.The predominant fatty acids in chicken meats of all treatments were palmitic and stearic (18:0) acids as SFA, oleic acid as MUFA, and LA and arachidonic acid (20:4n-6) as PUFA. Oleic and palmitic acids were the most abundant fatty acids in the various meats under analysis. Pasture consumption had little effect on the fatty acid profile of broiler meats. This is not completely unexpected, since the levels of pasture intake (in terms of DM) in birds with access to the legume-based pastures were observed to be low (see section 2.1). In addition, pasture intake did not reduced the levels of the cereal-based diets consumed but rather increased it. Although pastures presented LA/ALA ratios below 0.40, meat from free-range broilers had a much higher n-6/n-3 ratio (11.3-12.9) that was not affected by pasture intake (Table 2.9). It is known that ALA present in pasture is in the esterified form in structural lipids, including galactolipids from chloroplasts (Gurr, 1984). Therefore, it is possible that the broiler digestive system may not be able to digest structural lipids and/or may lack the required galactolipase activity to free ALA from galactolipids. Although consumption of both pastures did not affect the percentages of the major represented

fatty acids (P>0.05), the intake of the *T. repens*-based pasture reduced (P<0.01) the percentage of LA in broiler meat. This effect could be due to the reduced proportion of LA in the pastures and to slightly higher intakes observed in birds foraging in the *T. repens*-based pasture (data not shown). Interestingly, a seasonal effect (P<0.001) was observed on the content of LA, with higher percentages of the fatty acid being observed in broiler meat from the autumn experiment.

Access to the *T. subterraneum* and *T. repens*-based pastures may have contributed to alter the levels of the less represented 18:1 isomers and ALA fatty acids in meat from free range pastured broilers. The identified 18:1 isomers peak, which eluted after the 18:1 cis-9 fatty acid, suggests the presence of several *cis* and *trans* 18:1 isomers in the meat. The increased proportion of 18:1 isomers in meat from animals with access to pastures is unexpected and only future work utilizing longer columns (100 m) of highly polar stationary phase, will allow the efficient separation of these isomers. Although the proportion of 18:1 isomers was increased (P<0.001) in meat from birds with access to the pastures, the levels of the ALA in broiler meat were reduced (P<0.01) as a consequence of pasture intake. This is surprising since both legume-based pastures presented higher levels of ALA when compared with the cereal-based diet. This observation suggests a larger conversion of ALA to its derivatives in free-range broilers, which may result from the lower contents of LA in these animals, a well known competitor of ALA in the metabolism of the two essential fatty acid families as discussed below. Levels of EPA (P<0.01) and 22:4n-6 (P<0.001) were influenced by the combined effect of season and treatment. Although EPA levels were not affected by pasture intake in autumn, in spring EPA percentages were higher in meat from broilers consuming the leguminous biomass. Interestingly, the higher levels of EPA in the spring parallel a reduction in the content of ALA and LA in broiler meat. These results suggest that, as a consequence of low LA levels in broiler tissues, ALA is more effectively desaturated and elongated resulting in higher levels of EPA (Leece & Allman, 1996). However, the levels of EPA, DHA and 22:5n-3 in the free-range broiler meat are much lower when compared with the percentages of the long-chain n-3 fatty acids reported in meat originated in birds supplemented with 2-4% of fish oil (López-Ferrer *et al*., 2001a). Finally, pasture intake influenced the levels of 22:2n-6 and 22:4n-6 in broiler meat, although pasture species significantly influenced the type of response in terms of fatty acid

accumulation. Therefore, while the *T. subterraneum*-based pasture induced an increase in the percentages of 22:2n-6, the *T. repens* pasture promotes an increase in the percentage of 22:4n-6, although restricted to the spring experiment. Overall, the data suggests that low levels of pasture intake (<5% DM) did not contribute to improve the levels of ALA in breast meat, while desaturation and elongation of this fatty acid precursor may contribute, in a certain degree, to the synthesis of its long-chain family derivatives. Therefore, these results indicate that free access to high-quality pastures for free range pastured broilers with a cereal-based feed available *ad libitum*, is unable to substantially improve the n-3 fatty acids of broiler meat. Under these circumstances, direct supplementation with long-chain PUFA may be a more straightforward route to improve meat nutritive value.

Table 2.8 Fatty acid composition of breast meat of broilers fed *ad libitum* with a cereal-based feed without access to pasture (NP) or foraging in *Trifolium repens* (*Tr*P) or *T. subterraneum* (*Ts*P) based pastures, during spring and autumn.

	Autumn			Spring			SEM	Treatment		Season	T×S
	NP	*Tr*P	*Ts*P	NP	*Tr*P	*Ts*P		NP *vs* P	*Tr vs Ts*		
14:0	0.34	0.33	0.33	0.37	0.34	0.34	0.016	ns	ns	ns	ns
14:1	0.06	0.06	0.05	0.06	0.05	0.07	0.005	ns	ns	ns	ns
15:0	0.09	0.12	0.10	0.08	0.07	0.07	0.019	ns	ns	*	ns
15:1	0.06	0.09	0.08	0.02	0.02	0.02	0.021	ns	ns	*	ns
16:0	23.6	24.0	24.2	24.2	24.3	24.6	0.402	ns	ns	ns	ns
16:1n-7	2.46	2.56	2.23	2.39	2.24	2.57	0.146	ns	ns	ns	ns
17:0	0.12	0.14	0.13	0.14	0.14	0.13	0.005	ns	ns	ns	ns
17:1	0.15	0.21	0.20	0.25	0.22	0.31	0.058	ns	ns	ns	ns
18:0	11.5	11.9	11.8	12.5	12.1	12.1	0.331	ns	ns	ns	ns
18:1ism[1]	2.68	3.18	3.12	3.04	3.38	3.41	0.153	***	**	*	ns
18:1n-9	27.2	27.1	27.2	26.6	26.8	26.6	0.810	ns	ns	ns	ns
18:2n-6	17.8	16.8	17.4	16.6	15.3	15.7	0.442	**	**	***	ns
18:3n-3	0.45	0.41	0.40	0.41	0.35	0.34	0.023	**	*	**	ns
18:3n-6	0.12	0.11	0.11	0.13	0.12	0.12	0.013	ns	ns	ns	ns
20:0	0.08	0.09	0.08	0.08	0.10	0.08	0.008	ns	ns	ns	ns
20:1n-9	0.27	0.26	0.24	0.28	0.27	0.26	0.015	ns	ns	ns	ns
20:2n-6	0.64	0.63	0.57	0.67	0.72	0.75	0.047	ns	ns	*	ns
20:3n-3	0.02	0.02	0.02	0.03	0.04	0.04	0.005	ns	ns	***	ns
20:3n-6	0.94	0.87	0.84	1.69	1.03	1.06	0.320	ns	ns	ns	ns
20:4n-6	7.23	7.39	7.40	6.68	7.08	6.61	0.508	ns	ns	ns	ns
20:5n-3	0.16^{bc}	0.14^{bc}	0.12^{c}	0.17b	0.23^{a}	0.23^{a}	0.015	ns	ns	***	**
22:2n-6	0.06	0.08	0.07	0.07	0.08	0.09	0.007	*	ns	ns	ns
22:4n-6	2.10^{b}	1.84^{b}	1.66^{b}	1.82^{b}	3.11^{a}	2.91^{a}	0.231	*	*	***	***
22:5n-3	0.75	0.65	0.61	0.66	0.79	0.74	0.052	ns	ns	ns	ns
22:6n-3	1.05	0.96	1.01	0.93	1.07	0.91	0.071	ns	ns	ns	ns

[1]18:1 isomers

Table 2.9 Lipids and cholesterol contents (mg g^{-1} meat), selected sums of fatty acids (% w/w) and nutritional ratios in breast meat of broilers fed *ad libitum* with a cereal-based feed without access to pasture (NP) or foraging in *Trifolium repens* (*Tr*P) or *T. subterraneum* (*Ts*P) based pastures, during spring and autumn.

	Autumn			**Spring**			**SEM**	**Treatment**		**Season**	**T×S**
	NP	***Tr*P**	***Ts*P**	**NP**	***Tr*P**	***Ts*P**		**NP *vs.* P**	***Tr vs. Ts***		
Lipids	10.2	9.14	8.68	11.0	10.3	9.80	0.582	*	ns	*	ns
Cholesterol	0.50	0.51	0.49	0.50	0.51	0.51	0.014	ns	ns	ns	ns
Partial sums											
SFA	35.8	36.6	36.7	37.4	37.1	37.2	0.573	ns	ns	ns	ns
MUFA	30.2	30.3	30.0	29.6	29.6	29.8	0.920	ns	ns	ns	ns
PUFA	31.3	29.9	30.2	29.9	29.9	29.6	1.051	ns	ns	ns	ns
n-3	2.42	2.18	2.16	2.20	2.47	2.26	0.129	ns	ns	ns	ns
n-6	28.7	27.4	27.7	27.7	27.4	27.3	1.031	ns	ns	ns	ns
Ratios											
P/S	0.88	0.82	0.83	0.81	0.81	0.80	0.036	ns	ns	ns	ns
n-6/n-3	12.1	12.8	12.9	12.9	11.3	12.2	0.490	ns	ns	ns	ns

Table 2.10 Tocopherols and tocotrienols contents (μg g^{-1} meat) in chicken breast meat of broilers fed *ad libitum* with a cereal-based feed without access to pasture (NP) or foraging in *Trifolium repens* (*Tr*P) or *T. subterraneum* (*Ts*P) based pastures, during spring and autumn.

	Autumn			**Spring**			**SEM**	**Treatment**		**Season**	**T×S**
	NP	***TrP***	***TsP***	***NP***	***TrP***	***TsP***		**NP *vs.* P**	***Tr vs. Ts***		
α-Tocopherol[1]	5.75	6.10	5.72	5.52	6.17	5.18	0.350	ns	ns	ns	ns
β-Tocopherol	0.04	0.04	0.03	0.06	0.05	0.05	0.003	ns	ns	***	ns
γ-Tocopherol[2]	0.71	0.77	0.70	0.49	0.50	0.45	0.027	ns	ns	***	ns
γ-Tocotrienol	0.15	0.15	0.11	0.16	0.11	0.18	0.038	ns	ns	ns	ns

[1] Co-eluted with small amounts of α-tocotrienol; [2] Co-eluted with small amounts of β-tocotrienol.

Pasture intake had no impact ($P>0.05$) on the meat total cholesterol concentration (Table 2.9). In contrast, meat glycerol lipids (or non sterol lipids) were lower in birds with access to pastures when compared to those without access to pasture. However, all chicken meats are lean, based on the Food Advisory Committee (1990) criteria (<5% fat), and depict median contents of total cholesterol (0.49-0.51 mg/g), when compared with those reviewed by Chizzolini *et al.* (1999) for beef. α-Tocopherol, which co-eluted in these meats with small amounts of α-tocotrienol, was the major vitamin E homologue detected in breast meats (Table 2.10). In addition, small contents of γ-tocopherol, which co-eluted with a minor proportion of β-tocotrienol, β-tocopherol and γ-tocotrienol were also identified. In contrast, although the pastures presented detectable levels of δ-tocopherol (0.53-1.00 μg g-1 dry matter) and δ-tocotrienol (2.30-7.71 μg g-1 dry matter), these diterpenes were not detected in any of the meat samples analyzed. The prevalence of α-tocopherol in meat is well known and is due to the more than tenfold preference of the tocopherol-binding protein for α-tocopherol, relative to γ-homologues, which are the most common vitamin E molecules in plant foods (Decker et al., 2000). Finally, the levels of vitamin E compounds in meat were not affected ($P>0.05$) by pasture intake, although a seasonal variation ($P<0.001$) on the levels of β- and γ-tocopherols were observed (Table 2.10). In addition, although the pastures presented significant levels of β-carotene (2.96-21.4 μg g-1 dry matter), this lipid-soluble antioxidant pro-vitamin was not detected in any of the meat samples analyzed, which may be due to their lower fat content.

2.2.3.3 Comparison of fatty acid composition, cholesterol, tocopherols and tocotrienols in meat from conventional (Ross) and free-range (Lab) broilers

Today, in Europe and the United States, a higher proportion of consumers are interested in broiler specialty products derived from free-range production systems. In Europe, these systems use slower growing genotypes slaughtered at day 81 fed on cereal-based diets and with a vestigial ingestion of grass biomass. However, to our knowledge, it is still unknown what could be the influence of the combined effect of genotype, age and production system in the fatty acid profile of meat from these less intensive production systems, especially when compared with meat derived from animals of the conventional intensive production system, which use fast growing genotypes and slaughtering between days 35 to

42. Here, the fatty acid profile of meat from broilers of these two production systems under practice (Ross, conventional; and Lab, free-range) was compared. The data, presented in Table 2.11, confirms that there are considerable differences in the fatty acid profile of the two meats under analysis. In both meats, the predominant fatty acid was palmitic acid, followed by oleic acid. However, the two fatty acids were more represented in meat from the free-range broilers. As expected, in both meats the precursor of the n-6 fatty acid family, LA, predominates in relation to the precursor of the n-3 family, ALA, although both PUFA were present in higher percentages in meat from the fast growing genotype. While EPA and 20:3n-3 predominate in meat from the conventional broilers, DHA was more abundant in meat from the commercial free-range birds. In relation to the long-chain n-6 fatty acids, arachidonic acid predominates in meat from the slower growing genotype, while 22:4n-6 was more abundant in breast meat from the Ross genotype. The SFA ($P<0.001$) and MUFA ($P<0.01$) contents of meat from the free-range broilers were higher when compared with meat from the fast growing genotype, as shown in Table 2.11. Accordingly, fast growing birds presented breast meat with higher percentages ($P<0.001$) of PUFA. However, the n-6/n-3 ration was not different between the two meats, although the fast growing genotype presented breast meat with higher percentages ($P<0.01$) of both n-6 and n-3 fatty acids (Table 2.11). Taken together these data suggest that slower growing genotypes raised under free-range production systems may not originate meat with higher nutritional quality. Here, it was shown that meat from intensively grown birds slaughter at day 35 presented higher levels of PUFA and n-3 fatty acids when compared with the commercial free-range broilers.

Table 2.11 Lipids and cholesterol contents (mg g^{-1} meat), fatty acid composition and selected sums of fatty acids (% w/w) and nutritional ratios in commercial chicken breast meat from broilers of the Ross genotype, grown under a conventional intensive system and slaughter at d 35 (Ross), or from a slow growing genotype produced under the EU free range system with slaughtering at d 81 (Lab).

	Lab	Ross	SEM	Significance
Lipids	9.08	8.01	0.611	ns
Cholesterol	0.48	0.56	0.014	***
Fatty acids				
14:0	0.38	0.40	0.013	ns
14:1	0.06	0.06	0.006	ns
15:0	0.13	0.11	0.010	ns
15:1	0.02	0.00	0.005	*
16:0	25.2	23.3	0.432	***
16:1n-7	1.76	1.87	0.114	ns
17:0	0.18	0.17	0.005	*
17:1	0.30	0.15	0.053	*
18:0	12.5	11.5	0.234	***
18:1ism	2.91	3.26	0.079	***
18:1n-9	24.9	21.8	0.650	***
18:2n-6	17.3	21.2	0.412	***
18:3n-3	0.52	0.91	0.056	***
18:3n-6	0.10	0.14	0.013	*
20:0	0.36	0.10	0.195	ns
20:1n-9	0.20	0.39	0.018	***
20:2n-6	0.54	1.48	0.042	***
20:3n-3	0.03	0.19	0.009	***
20:3n-6	0.72	1.46	0.051	***
20:4n-6	8.38	5.76	0.354	***
20:5n-3	0.13	0.39	0.021	***
22:2n-6	0.04	0.12	0.011	***
22:4n-6	1.60	3.73	0.204	***
22:5n-3	0.90	0.88	0.053	ns
22:6n-3	0.91	0.59	0.064	***
Partial sums				
SFA	38.8	35.6	0.491	***
MUFA	27.2	24.3	0.722	**
PUFA	31.1	36.9	0.782	***
n-3	2.47	2.96	0.114	**
n-6	28.6	33.9	0.681	***
Ratios				
P/S	0.81	1.04	0.029	***
n-6/n-3	11.7	11.5	0.316	ns

While total lipids are present at similar levels in both meats, Ross meat was significantly more abundant ($P<0.001$) in total cholesterol (Table 2.11). Cholesterol is an important molecule that has roles in membrane structure as well as being precursor for the synthesis of molecules such as steroid hormones, vitamin D and bile acids. Cholesterol can be obtained directly from the diet, or it can be synthesized in cells from two-carbon acetate groups of acetyl-CoA. Because the synthetic pathway is under feedback control from dietary cholesterol, the percentage of cholesterol arising from *de novo* biosynthesis depends upon the amount of cholesterol in the diet. Even when cholesterol intake is very low, *de novo* biosynthesis will enable the production of the cholesterol required to supply the large variety of biological processes in which this molecule is involved. Therefore, although the data suggests that fast growing and younger birds present higher levels of cholesterol it is unknown if these results are the consequence of high cholesterol levels in the diet or result from a genotype influence *per se*. Finally, in both meats α-tocopherol, which co-eluted with small amounts of α-tocotrienol, was present at similar levels ($P>0.05$) and was the most abundant compound with vitamin E activity, in accordance with the putative supplementation of the broiler diets with significant and similar levels of exogenous α-tocopherol acetate (Table 2.12). In addition, small amounts of γ-tocopherol, which co-eluted with minor amounts of β-tocotrienol, β-tocopherol and γ-tocotrienol were also determined. In contrast, the diterpenes δ-tocopherol and δ-tocotrienol were not detected in any of the meat samples analyzed. There were no significant differences ($P>0.05$) for the minor vitamin E compounds between Lab and Ross meats, with the exception of γ-tocopherol, which was higher ($P<0.001$) in Ross meat.

Table 2.12 Tocopherols and tocotrienols contents (μg g^{-1} meat) of commercial chicken breast meat from broilers of the Ross genotype, grown under a conventional intensive system and slaughter at d 35 (Ross), or from a slow growing genotype produced under the EU free range system with slaughtering at d 81 (Lab).

	Lab	**Ross**	**SEM**	**Significance**
α-Tocopherol[1]	4.30	4.33	0.253	ns
β-Tocopherol	0.05	0.05	0.004	ns
γ-Tocopherol[2]	0.41	0.88	0.046	***
γ-Tocotrienol	0.17	0.17	0.058	ns

[1] Co-eluted with small amounts of α-tocotrienol; [2] Co-eluted with small amounts of β-tocotrienol.

In conclusion, in this study lower levels of pasture intake (<5% DM) were shown to have a low impact on fatty acid and vitamin E homologue profiles of meat from free-range broilers, suggesting that meat properties were more dependent on the composition of the cereal-based feed available for *ad libitum* consumption. However, animals with access to pastures presented lower levels of the n-6 and n-3 fatty acid precursors, LA and ALA, respectively, in breast meat. In addition, in spring the levels of EPA in breast meat were significantly higher in animals consuming pastures, which suggest a higher conversion of ALA into EPA in these birds. Finally, when compared with meat from slow growing genotypes obtained in low intensive production systems with slaughtering at day 81 (Lab), meat from Ross birds raised intensively and slaughtered at day 35 presented higher nutritional indices.

CHAPTER 3 CROP BETA-GLUCANASE ACTIVITY LIMITS THE EFFECTIVENESS OF A RECOMBINANT CELLULASE USED TO SUPPLEMENT A BARLEY-BASED FEED FOR FREE-RANGE BROILERS

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ABSTRACT

The supplementation of diets rich in soluble polysaccharides for simple stomach animals with microbial cellulases and hemicellulases decreases digesta viscosity and promotes broiler performance. In contrast, recent experiments suggest that polysaccharidases are inefficient for improving the nutritive value of pasture biomass used by free-range broilers. However, the feasibility of using cellulases and hemicellulases to improve the utilization of cereal-based feeds by pastured poultry remains to be established. A study was undertaken to investigate the capacity of a recombinant cellulase from *Clostridium thermocellum*, to improve the nutritive value of a barley-based feed for free-range pastured broilers of the RedBro Cou Nu × RedBro M genotype. The data show that supplementation of a barley based diet with a recombinant beta-glucanase had no effect on the performance of free-range broilers, foraging in legume-based diets from days 28-56. In addition, the results confirm that the lack of effect of the recombinant enzyme in improving the nutritive value of the barley-based feed does not result from enzyme proteolysis or inhibition in the GI tract. Significantly, beta-glucanase activity was identified in the crop of non-supplemented animals. The data suggest that endogenous cellulases are originated both from the barley-based feed and from the crop microflora. The results presented here suggest that in older birds of slow-growing genotypes associated with free-range production systems, previously unknown sources of beta-glucanases, such as the feed and microbial symbiotic microflora, can affect the effectiveness of exogenous enzymes added to the feed.

***Key words*:** recombinant cellulase, free-range broilers, feed enzymes

3.1 INTRODUCTION

In general, inclusion of exogenous 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 (Chesson, 1993; Bedford, 2000). 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 (White *et al*., 1981; Fengler & Marquardt, 1988; Bedford *et al*., 1991; Bedford & Classen, 1992). 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 (Bedford & Morgan 1996; Apajalahti & Bedford, 1999). 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). The action of one or a conjunction of the above-mentioned effects may depend on the type of animal, diet and exogenous enzyme used.

Recently consumer interest in specialty poultry products derived from free-range or organic production systems has been steadily increasing, both in the United States and in Europe (Fanatico *et al.*, 2006). Under these systems, animals have access to the outdoor to promote foraging, feed selection and activity, thus improving birds' general welfare. In Europe, poultry used under these systems, including the European organic production programs, are derived from slow-growing genotypes that are slaughtered at later stages of the growth cycle, generally between weeks 11 and 14 of age. It has been suggested that slow-growing birds are more adapted to less intensive production systems while the quality of their meat is more appropriated for a specialty or gourmet market (Gordon & Charles, 2002). In a recent study we showed that a complex mixture of cellulases and hemicellulases is unable to promote the nutritive value of legume-based pastures used by free-range broilers of a slower-growing genotype (Ponte *et al*., 2008a; section 2.1).

Although exogenous plant cell wall hydrolases may not be effective for releasing more energy from plant biomass, it remains to be established if these biocatalysts permit the incorporation of higher percentages of cereals rich in soluble NSP in diets for free-range broilers. Specifically, it is still unknown if cellulases and hemicellulases can improve the nutritive value of cereal-based diets for free-range pastured poultry of slower-growing genotypes slaughter at later stages of growth. In addition, it is well established that individual recombinant enzymes are as efficient as complex enzyme mixtures to decrease the digesta viscosity of broilers of fast-growing genotypes raised under the current intensive production systems that are fed on cereals rich in soluble NSP (Philip *et al*., 1995, Fontes *et al*., 2004). However, the possibility of using individual recombinant enzyme to depolymerise the anti-nutritive β-glucans found in barley-based diets for free-range broilers of slow growing genotypes remains to be investigated.

The objective of this work was to evaluate the capacity of a bifunctional recombinant derivative of *Ct*Lic26A-Cel5E from *Clostridium thermocellum*, consisting on the enzyme two catalytic modules expressed as an individual entity; to enhance nutritive value of a barley based diet for free-range pastured birds of a slow-growing genotype, from days 28-56.

3.2 MATERIAL AND METHODS

3.2.1 Enzyme preparation

*C. thermocellum Ct*Lic26A-Cel5E is a thermostable bi-functional enzyme containing β-1,3-1,4-glucanase (GH26) and β-1,4-cellulase (GH5) catalytic domains, in addition to two non-catalytic modules. The molecular architecture of *Ct*Lic26A-Cel5E and its truncated recombinant derivatives used in this study are presented in Figure 3.1. The enzyme contains an N-terminal GH26, followed by a second GH5 catalytic module, 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-Cel5-Cbm11, Lic26-Cel5, Lic26 and Cel5 were hyperexpressed in *Escherichia coli* following the protocols described by Taylor *et al*. (2005). The recombinant plasmids containing the four 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). *E. coli* cell free extracts containing the recombinant cellulase A (CelA) from *Ruminococcus albus* were prepared as described by Fontes *et al*. (1995).

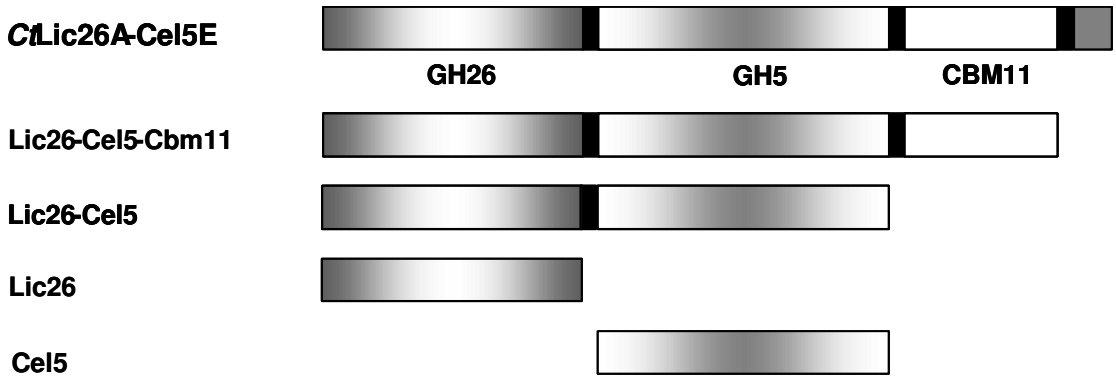

Figure 3.1 Molecular architecture of *Ct*Lic26A-Cel5E.
*Ct*Lic26A-Cel5E is a modular cellulase composed of an N-terminal family 26 glycoside hydrolase catalytic domain (GH26), an additional family 5 glycoside hydrolase catalytic domain (GH5), a family 11 carbohydrate-binding module (CBM11) and a C-terminal dockerin.

3.2.2 Animals, diets and management

The effect of a recombinant β-glucanase in the nutritive value of a barley-based feed for free-range pastured broilers raised between days 28 and 56 was evaluated in an experiment performed in the spring of 2004 at Herdade dos Esquerdos (039º 07.18' north,

007º 29.36' west, 318 m a.s.l.), Vaiamonte, Portugal. During the experiment, the average daily mean temperature was 13.2 ºC (mean of highest temperatures 19.0 ºC and of the minimum 7.4 ºC) with four days with rain and a total precipitation of 34 mm. One hundred and sixty 28-d-old males RedBro Cou Nu x RedBro M, vaccinated against Marek disease, were divided into 16 floorless portable metal outdoor pens (10 birds per pen/replicate), equalizing both the mean and the variance of body weight (BW). Previous to the commencement of the experiment animals were maintained in a conventional in-door facility following standard brooding procedures and fed on a typical maize soybean meal diet. At day 28, animals were transported to the experimental field and raised for a further 28 d in the outdoor, confined to the experimental pens that were kept in the pasture till slaughtered at d 56. The movable pens measured 1.7 m × 1.5 m × 0.5 m (0.255 m^2 per bird) and allowed birds to directly contact the legume-based pastures, promoting forage intake. Approximately one third of the top of each cage area was covered with transparent white-washed plastic to provide protection against harsh climate periods. Water and a barley-based feed were available *ad libitum* throughout the experiments and were provided in two automatic drinking nipples and in an individual hanging tube feeder, respectively. The composition of the basal diet used on this study (Table 3.1), which was provided in the pelleted form, was formulated to contain adequate nutrient levels as defined by the NRC (1994).

The birds were randomly assigned into one of the four treatments with 4 replicates of ten birds per treatment. The four treatments consisted on two levels of enzyme supplementation, no exogenous plant cell wall hydrolase (No enzyme) or supplementation with 4000 U/kg of the recombinant β-glucanase Lic26-Cel5 (Enzyme), and two types of pasture, consisting of an irrigated white clover (*Trifolium repens)* pasture (*Tr*P) or a rainfed subterranean clover (*Trifolium subterraneum)* pasture (*Ts*P), in a completely randomized experiment. Activity of the recombinant enzyme was determined as described below. At day 42, half-way through the experiment, samples of both pastures were collected from 1 m^2 paddocks, by cutting it at 3 cm above the ground, for proximate analysis that was performed as described below. To promote forage intake, the portable pens were moved daily so that birds could dispose of fresh herbage every day. Throughout the year a sheep flock was introduced in the pasture, when necessary, to keep the size of

the vegetation below 12-15 cm above ground. To avoid ingestion of sheep faecal material by birds, sheep faeces were removed from pasture previous to the movement of the pens. In order to avoid climate variations, the two pastures used on this experiment, which were installed in the autumn of 2002, were contiguous. The White clover pasture was irrigated during the dry summer season (june-september).

Table 3.1 Ingredient composition and calculated analysis of the cereal-based feed.

Ingredients	**%**
Barley	61.90
Soybean meal 42%	28.13
Soybean oil	6.28
Salt	0.28
Calcium carbonate	1.15
Dicalcium phosphate [1]	1.72
Choline 60%	0.08
DL-Methionine	0.17
Mineral and vitamin premix [1]	0.20
Calculated nutrient content	
Energy (MJ ME/kg DM)	12.12
Crude Protein (%)	18.40
Ether extract (%)	7.70
Crude fibre (%)	5.00

1 Contained 20% Ca and 18% P.
2 Mineral-vitamin premix provided the following per kilogram of diet: vitamin A, 9,000 IU; vitamin D3, 2,100 IU; vitamin E, 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, 100mg; Zn, 80 mg; Co, 0.2 mg; I, 1.0 mg; Se, 0.3 mg; monensin, 100 ppm.

Feed consumption and individual body weights were recorded weekly. 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 56, two birds per pen were slaughtered by an intravenous injection of an aqueous solution of 125 mg Tiopental Braun (Braun, Barcelona, Spain).

The size of the various GI compartments was measured and digesta was collected from different digestive compartments for posterior analysis. Levels of cellulase and hemicellulase activity in the GI tract were measured as described below. The proportion of forage and high-energy feed found in the crop was measured to estimate pasture consumption. Although chicken have been reported to feed on a wide range of macro-invertebrates living in the surface soil (Clark & Gage, 1996), the contribution of this behaviour to the nutrition of the free-range broilers was not quantified. In addition, at the end of the experiment, at day 56, six birds per pen were slaughtered at a commercial processing plant. The carcasses were refrigerated for 24 hours and weighed. Meat pH was measured as described by Sierra (1973).

3.2.3 Skin colour

The colour of breast skin was evaluated using a Minolta chroma meter CR-300. The readings were taken on equivalent positions of the carcasses. The tip of the chromameter-measuring head was placed flat against the surface of the skin. For each reading 3 measurements were performed and the final value for each animal is the average of those readings. Skin colour was expressed in the CIELAB dimensions of lightness (L), redness (a*) and yellowness (b*). After skin colour evaluation, carcasses were frozen at -20 ºC until posterior analysis.

3.2.4 Analytical procedures

Analyses for dry matter (DM), ether extract, crude protein and dietary fibre were performed according to the Association of Official Analytical Chemists (1980) methods. Enzyme activity was determined at 40 ºC by measuring reducing sugar released, following the method described by Taylor *et al*. (2005), using barley β-glucan (Megazyme®, Ireland) as the substrate. One unit of enzyme activity is defined as the amount of enzyme required to release one µmole of product per min. Digesta samples were centrifuged and the supernatant was analysed for beta-glucanase activity. 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. Activity was detected after 16 hours incubation at 37 ºC through the Congo Red assay plate, as described in Ponte *et al.* (2004b) and Mourão *et al*. (2006). Due to the presence of high levels of reducing sugars in digesta samples, the quantification of β-glucanase activity in the feed and in the crop samples was determined following the Azo-barley glucan method, using azo-barley glucan (Megazyme®, Ireland) as substrate, following the manufacturers protocol. Zymogram analysis was performed as described by Fontes *et al*. (2004). Briefly, digesta proteins were separated through SDS/PAGE in 12 % 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, for 15 min and washing with 1 M NaCl until excess dye was removed. Areas of enzyme activity appeared as colourless zones in a dark blue background after a quick wash in a 0.5% (v/v) solution of acetic acid. Resistance of the *Ct*Lic26A-Cel5E truncated derivatives to proteolysis was tested essentially as described by Dias *et al*. (2004). Briefly, the recombinant proteins were incubated with porcine pancreatine (Sigma, St. Louis, USA) for 3 hours at 37ºC and retained enzyme activity was measured using the reducing sugar assay described above. To evaluate the consequence of the proteolytic attack on the molecular integrity of the recombinant proteins, samples were also analysed through SDS-PAGE. For viscosity measurement of the duodenum content, 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 24ºC.

3.2.5 Statistical analysis

Statistical analysis was conducted by analysis of variance using SAS with the GLM procedure (SAS Institute, 2004). The experimental unit considered was the pen. In relation to the animal performance and meat physical properties data, the model considered the effects of the type of pasture consumed, enzyme supplementation and the interactions between the two effects. In the GI tract measurement data, from *Ts*P birds, the model considered the effect of enzyme supplementation. For these parameters the experimental unit considered was the bird. Unless otherwise stated, differences were considered significant when $P<0.05$.

3.3 RESULTS AND DISCUSSION

3.3.1 Enzyme selection

Exogenous enzymes used for supplementing diets for simple stomach animals need to retain catalytic activity in the conditions prevailing in the GI tract. Specifically, the microbial biocatalysts need to resist to the proteolytic inactivation by endogenous digestive enzymes and remain active at the pH and temperatures of the digestive system. The modular thermostable enzyme from *C. thermocellum*, termed *Ct*Lic26A-Cel5E, contains an N-terminal family 26 glycoside hydrolase catalytic domain (GH26), an additional internal family 5 glycoside hydrolase catalytic module (GH5), a family 11 carbohydrate-binding module (CBM11) and a C-terminal dockerin (Taylor *et al*., 2005;

Figure 3.1). Linker sequences rich in hydroxyl aminoacids separate the various domains of *Ct*Lic26A-Cel5E. To select an adequate recombinant β-glucanase for supplementing barley-based diets for poultry, the resistance of the various truncated derivatives of *Ct*Lic26A-Cel5E to proteolytic inactivation was determined. The data, presented in Figure 3.2 (Panel A), demonstrate that incubation of Lic26-Cel5-Cbm11, Lic26-Cel5, Lic26 and Cel5 with pancreatic proteases for a 3 hour period did not affect the catalytic activity of

the various Clostridial enzymes, which remain essentially unchanged. In contrast, under identical experimental conditions cellulase A (CelA) from *Ruminococcus albus*, which is known to be susceptible to proteolytic inactivation (Fontes *et al*., 1995), retained around 6 % of its initial activity. Together, the data suggests that in common with a large variety of microbial cellulases, the Clostridial recombinant enzymes are resistant to proteolytic inactivation. It is, however, unknown if retention of enzymatic activity is accompanied by retention of the recombinant enzymes´ molecular architectures. To investigate the molecular structure of the Clostridial cellulases following incubation with the animal proteases, SDS-PAGE analysis was used to determine the number and size of the polypeptides resulting from proteolytic attack. The data, presented in Figure 3.2 (Panel B), demonstrates that although the molecular integrity of Lic26 and Cel5 is not affected by the action of pancreatic proteases, the bi-modular and tri-modular derivatives of *Ct*Lic26A-Cel5E were transformed in their two and three constituting modules. Taken together, the data suggest that although proteases are unable to affect the integrity of the various modules of *Ct*Lic26A-Cel5E *per se*, the enzymes are prone to proteolysis at the linker sequences that separate the various domains of the modular enzyme. Since the transformation of Lic26-Cel5 into its two molecular constituents, Lic26 and Cel5, has not affected the efficiency of barley β-glucan enzymatic degradation, the bi-modular truncated derivative of *Ct*Lic26A-Cel5E, Lic26-Cel5, was selected for supplementing a barley-based feed available *ad libitum* for free-range pastured broilers.

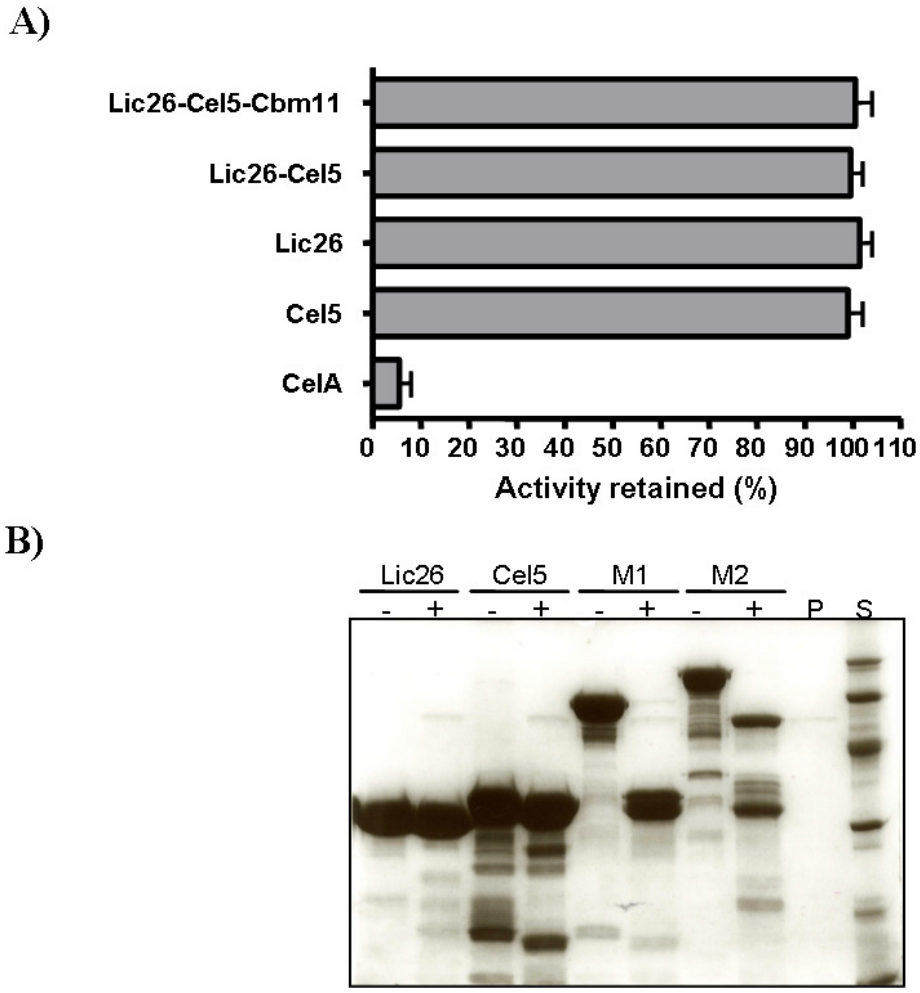

Figure 3.2 Resistance of the truncated derivatives of CtLic26A-Cel5E to proteolysis.
The four truncated derivatives of *Ct*Lic26A-Cel5E were incubated with pancreatic proteases. Retained activity (Panel A) and molecular integrity (Panel B) were evaluated after a 3 hour incubation period. In panel A CelA refers to the cellulase A from *Ruminococcus albus* that is susceptible to proteolysis. In Panel B, the recombinant enzymes were incubated (+) or not incubated (-) with the pancreatic proteases and the molecular integrity of the enzymes evaluated through SDS-PAGE. M1 and M2 refer to Lic26-Cel5 and Lic26-Cel5-Cbm11, respectively. The lanes P and S in Panel B, refer to the pancreatic proteases and the low molecular mass protein markers, respectively.

3.3.2 Bird performance

It remains unknown if β-glucanase supplementation can improve the nutritive value of cereal-based diets for free-range pastured broilers of slower-growing genotypes. Here, the effect of supplementing a barley-based feed with a recombinant bi-functional cellulase on

the performance of free-range broiler chicks foraging on legume-based pastures was evaluated. The enzyme is a truncated derivative of the thermostable *Ct*Lic26A-Cel5E from *C. thermocellum* termed Lic26-Cel5 and contains two catalytic domains expressing β-1,3-1,4-glucanase (GH26) and β-1,4-cellulase (GH5) specific activities, respectively. In advance of supplementation, the recombinant protein was purified through metal chelate affinity chromatography. Protein purity was assessed through SDS-PAGE analysis such as it is exemplified in Figure 3.2, Panel B. The barley-based feed was prepared, pelleted, supplemented with the recombinant enzyme and was available *ad libitum* for free-range broiler chicks foraging in legume-based pastures through days 28 to 56. The mortality rate during the experiment was low (6.2 %) and was not related with the treatments.

Legume-based permanent pastures are particularly well adapted to the Mediterranean region, which is extremely biodiverse in species of this plant family. In the autumn of 2002, 2 ha of a rainfed subterranean clover pasture and 1.5 ha of an irrigated white clover pasture were installed at Herdade dos Esquerdos. As expected, the grass composition of the pastures reflected the seed mixture and included annual ryegrass (*Lolium multiflorum)* and perennial ryegrass (*Lolium perenne*), that were co-seeded with subterranean clover and white clover, respectively. Other volunteer legume species consisted mainly of Balansa clover (*T. michelianum*) and Persian Clover (*T. ressupinatum*). The nutritive value of the herbage, which changes according to the time of the year, was determined at the time of the experiment in the spring of 2004. The data, presented in Table 3.2, confirms that both pastures displayed relatively high crude protein contents as a consequence of the predominance of leguminous species. However, DM percentages were always relatively low (see Table 3.2) and it is clear that fibre remains the main organic component of the pasture.

Table 3.2 Chemical composition of the legume-based pastures used by the free-range broilers in this experiment (% DM).

	T. subterraneum	**T. repens**
Dry matter	14.60	19.56
Crude protein	21.66	22.35
Ether extract	2.05	2.14
Crude fibre	22.66	19.09
NDF	40.02	40.15

The evolution of body weights and weight gains, total feed intake and feed conversion ratios throughout the experiment are summarized in Table 3.3. The data showed that final body weights were not affected by pasture type or enzyme supplementation, although there is an interaction between pasture type and supplementation relative to the weight gain at the first week. In addition, differences in feed intake and feed conversion ratio between the various experimental groups were not significant.

Moreover, as displayed in Table 3.4, the relative size and length of the different sections of the GI tract were not affected by the inclusion of the exogenous enzyme in the diet. Dietary fibre causes a significant enlargement in the GI tract of birds, as a result of an increased muscular development of the small intestine to cope with the large levels of non-digestible material and an increase in the microbial activity in the hindgut (Brenes *et al.*, 2002). Therefore, the data suggested that enzyme supplementation had no influence in the degree of hydrolysis of fibre from both the pasture biomass and the barley-based feed. Taken together, the results suggest that the inclusion of the recombinant thermostable cellulase from *C. thermocellum*, termed Lic26-Cel5, was unable to improve the performance of pastured broilers of a slow-growing genotype fed on a barley-based feed *ad libitum*.

Table 3.3 Growth performance of free-range broilers fed on a barley-based feed supplemented (enzyme) or not supplemented (no enzyme) with a mixture of cellulases and hemicellulases, foraging in *Trifolium subterraneum* (*Ts*P) or *T. repens* (*Tr*P) based pastures.

	***Tr*P**		***Ts*P**		**SEM**	**Significance**		
	No Enzyme	**Enzyme**	**No Enzyme**	**Enzyme**		**P**	**E**	**PxE**
Body Weight (g)								
28d	845.5	847.5	839	844.8	7.36	ns	ns	ns
35d	1142.5	1135.3	1099.0	1135.8	12.81	ns	ns	ns
42d	1556.8	1519.3	1499.3	1540.5	25.64	ns	ns	ns
49d	1825.3	1819.3	1818.7	1865.3	35.28	ns	ns	ns
56d	1978.8	1977.5	1989.7	2050.0	42.34	ns	ns	ns
Weight Gain (g)								
28-35d	297.0	287.8	260.3	291.0	9.18	ns	ns	*
35-42d	414.3	384.3	400.3	404.8	16.29	ns	ns	ns
42-49d	268.8	300.0	319.3	325.0	37.88	ns	ns	ns
49-56d	153.5	158.5	170.7	184.5	24.84	ns	ns	ns
28-56d	1133.3	1130.0	1150.7	1205.3	41.01	ns	ns	ns
Feed Intake[1] (g)								
28-56d	3725.0	3761.0	3744.0	4000.8	109.09	ns	ns	ns
FCR[1]								
28-56d	3.396	3.381	3.273	3.439	0.1690	ns	ns	ns

Significance: ns, $P>0.05$; *, $P<0.05$; SEM, standard error of mean.[1] Feed intake and feed conversion are relative to the cereal-based feed.

Table 3.4 Relative weight and length of GI tract of free-range broilers fed on a barley-based feed foraging in a *Trifolium subterraneum* (*Ts*P) based pasture.

	***Ts*P**		**SEM**	**Significance**
	No Enzyme	**Enzyme**		
Relative Weight (g/100g BW)				
Crop	0.401	0.358	0.0203	ns
Gizzard	1.597	1.723	0.1089	ns
Liver	3.440	3.001	0.2050	ns
Pancreas	0.252	0.217	0.0166	ns
Relative Length (cm/100g BW)				
Duodenum	1.722	1.706	0.0963	ns
Jejunum+Ileum	10.339	9.581	0.6292	ns
Caecum	1.246	1.240	0.0596	ns

Significance: ns, $P>0.05$; SEM, standard error of mean.

Considering the theoretical suboptimal environmental conditions to which the free- range chicken were subjected, when compared with birds housed indoors, it is interesting to verify that the growth rate achieved by the pastured broilers during spring is at the levels expected for the genotype RedBro Cou Nu x RedBro M (2079 g of BW at day 56 – Hubbard ISA management manual). Not surprisingly, however, feed conversion ratios were considerably higher than expected for this genotype (should be 2.1-2.2 at day 56), suggesting that animals can compensate growth at inappropriate temperatures by increasing feed intakes. This trial was developed in the spring when the weather was mild and did not fluctuate widely. Therefore, it is clear that more research is needed to evaluate the impact of periods such as the summer (very hot and dry) and the winter (cold and humid) on performance. In addition, differences in feed conversion ratios when compared with the values referred by the Hubbard ISA management manual may also result from the lower energetic concentration of the barley-based feed used on this study 12.12 MJ EMA/kg when compared the recommended 13.38 MJ EMA/kg as specified in the management manual.

Levels of forage intake were determined by evaluating the proportion of pasture and cereal-based feed found in the crops of sacrificed birds at the end of the experiment. The data revealed that grass biomass represent between 5.3-6.4 % on a DM basis or 17.3-21.7 % on a fresh basis, of the total feed intake in grazing animals, without significant

differences among treatments (data not shown). Although these values should be viewed with some caution, since they represent an estimate of the pasture consumption at a specific moment of the trial and forage consumption may have varied during the 28 days of the experiment and even during the same day, they represent a crude first estimate of biomass intake in free-range broilers. The recorded percentages of forage intake in this study are similar to the ones reported by Ponte *et al*. (2008a; section 2.1), in a similar experiment.

3.3.3 Meat physical properties

The influence of enzyme supplementation and pasture type in some important variables of the overall quality of poultry meat, such as carcass yield, meat pH and skin colour was investigated. The data, presented in Table 3.5, showed that neither enzyme supplementation nor pasture type influenced carcass yield and meat pH. This is not particularly surprising since exogenous enzymes are known to decrease the detrimental aspects associated with the ingestion of particular anti-nutritive factors, rather than affecting meat quality *per se*.

Table 3.5 Carcass yield (%), pH and breast skin colour of free-range broilers fed on a barley-based feed foraging in *Trifolium subterraneum* (*Ts*P) or *T. repens* (*Tr*P) based pastures.

	***Tr*P**		***Ts*P**		**SEM**	**Significance**		
	No Enzyme	**Enzyme**	**No Enzyme**	**Enzyme**		**P**	**E**	**PxE**
Carcass Yield	65.8	66.7	66.5	66.1	0.75	ns	ns	ns
pH	5.54	5.51	5.54	5.53	0.029	ns	ns	ns
Skin Color[1]								
L	90.5	89.3	93.3	89.8	1.25	ns	*	ns
a*	2.38	3.98	2.78	3.14	0.398	ns	*	ns
b*	-3.70	-2.12	-4.93	-4.66	0.812	*	ns	ns

Significance: ns, $P>0.05$; *, $P<0.05$; SEM, standard error of mean; [1] Skin colour was expressed in the CIELAB dimensions of lightness (L), redness (a*) and yellowness (b*).

However, it is possible that the action of feed enzymes may contribute to release a range of trapped bioactive molecules from the pasture biomass, such as xanthophylls, leading to a modification of meat colour. This effect may be more pronounced in pastured animals disposing a barley-based feed for *ad libitum* consumption, which is known to have a low concentration of meat colouring compounds. Therefore, to establish the feasibility of this hypothesis the influence of enzyme supplementation and pasture type in breast skin colour was evaluated. The results, presented as the CIELAB values of L (lightness), a (redness) and b (yellowness) are presented in Table 3.5. The data suggests that the interaction of enzyme and pasture type had no influence in colour meat parameters. Interestingly, enzyme supplementation with a recombinant microbial cellulase decreased meat L scores, indicating a more deeply pigmented skin. Animals receiving the exogenous feed enzyme displayed a considerable increase in the broiler carcasses redness (a), showing that the usually undesirable pink and red tones in the skin were more developed. Enzyme supplementation had no influence in skin b values, suggesting that the enzyme was not effective in releasing bioactive molecules from pasture involved in pigmenting the carcasses with yellow tones. Overall, the carcasses of all treatments had a very low pigmentation with yellow tones, resulting from the higher proportion of barley in the cereal-based feed and suggesting that pasture consumption at the levels verified under this experiment has a lower capacity to improve the b parameter of breast skin colour. However, birds foraging on the *T. repens* pasture displayed higher yellowness (b) scores, which may result from its higher proportion in carotenoid pigments. Taken together, the data suggests that enzyme supplementation of free-range pastured broilers contributes to improve the pigmentation of animal's carcasses especially with pink and red tones. In addition, the botanical composition of pastures may affect its capacity to change carcass colours, with *T. repens* based pastures being more effective in improving meat yellowness when compared with *T. subterraneum* pastures.

3.3.4 Recombinant β-glucanase stability *in vivo*

It was anticipated that the introduction of a microbial recombinant cellulases in a barley based feed available *ad libitum* for pastured broilers, could have contributed to decrease

the anti-nutritive properties associated with the ingestion of barley β-glucans. The data presented here suggests that the exogenous bi-modular enzyme had no effect in the performance of free-range broilers of a slow-growing genotype from days 28 to 56. It is presently recognized that the response of diet supplementation with cellulases and hemicellulases in terms of animal performance is not always positive and may vary with a range of factors such as animal age, microbial challenge, cereal genotype and growing conditions (Bedford, 2000). One possible explanation for the inability of the Clostridial enzyme to improve the nutritive value of the barley-based feed may be related with the lower concentration of soluble β-glucans in the barley used to prepare the cereal-based feed. Other experiments have shown that the level of β-glucans in barleys may vary with the cereal genotype or with the storage length and conditions (Fuente *et al*., 1998; Svihus *et al*., 1997), although it remains unknown if one or the conjunction of both these factors may have operate in this case. In addition, if pasture consumption can, in part, attenuate the detrimental effects associated with the intake of the barley-based feed and through which mechanism, remains to be established. Moreover, it has also been suggested that lack of exposure to the exogenous cellulases from day one of growth may limit the effectiveness of feed enzymes (Rosen, 2001; Bedford, 2002).

Following on the above discussion, the observed lack of response to enzyme supplementation could have resulted from enzyme inhibition and/or proteolysis during passage through the animal's GI tract. Therefore, to evaluate these possibilities, digesta samples collected from the various gastrointestinal compartments of animals foraging in the *T. subterraneum* pasture were tested for β-glucanase activity, using the Congo Red plate assay. The data, presented in Table 3.6, demonstrated that β-glucanase activity could be detected along the entire digestive tract in broilers fed on the barley-based feed supplemented with the *Clostridial* plant cell wall hydrolase, confirming *in vitro* results on the resistance of CtLic26A-Cel5E truncated derivatives to proteolysis. Therefore, a considerable percentage of the exogenous enzyme resist to the acidic and proteolytic conditions which are prevalent in some portions of the digestive tract. Unexpectedly, digesta samples collected from birds not receiving the recombinant enzyme presented considerable beta-glucanase activity, particularly in the crop – only four birds out of the 16 analysed did not present detectable beta-glucanase activity in the crop.

Table 3.6 Qualitative detection of beta-glucanase activity in digesta collected from the gastrointestinal compartments of 32 free-range broilers fed on a barley-based feed, supplemented (enzyme) or not supplemented (no enzyme) with a recombinant cellulase, foraging in *Trifolium repens* (*Tr*P) or *T. subterraneum* (*Ts*P) based pastures.

	Qualitative beta-glucanase activity [1]			
	Crop	**Gizzard**	**Duodenum**	**Caecum**
***Tr*P**				
Enzyme	+/+/+/+/+/+/+/+	+/+/+/+/-/+/+/+	+/+/+/+/-/+/+/+	+/+/+/+/+/+/+/+
No Enzyme	+/-/+/-/+/-/+/+	+/-/+/-/-/-/-/-	+/-/+/-/-/-/+/+	+/+/+/+/+/+/+/+
***Ts*P**				
Enzyme	+/+/+/+/+/+/+/+	+/-/-/+/-/+/-/+	+/+/-/+/+/-/+/-	+/+/+/+/+/+/+/+
No Enzyme	+/+/+/+/+/-/+/+	-/-/+/-/+/-/+/+	+/+/-/-/+/-/+/-	+/+/+/+/+/+/+/+

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

However, the size of the halos corresponding to beta-glucanase crop activity of non-supplemented animals was suggested to be sensibly smaller, as it can be observed in Figure 3.3 (Panel B). Together these data suggest that there is a considerable endogenous beta-glucanase activity in the GI tract of the free-range broilers, which could originate in enzymes present in the cereal feed (Bengtsson *et al*., 1990; Grosjean *et al*., 1999; Jeraci & Lewis, 1989) or in symbiotic crop microflora.

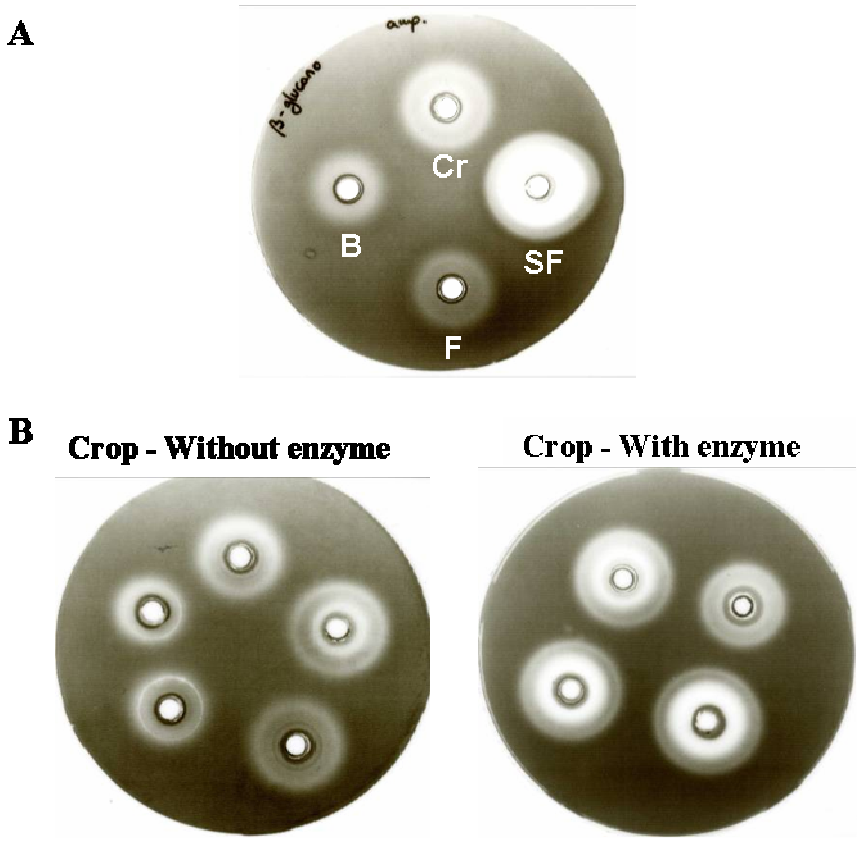

Figure 3.3 Detection of β-glucanase activity in the feed (Panel A) and crop contents (Panel B) of free-range broilers fed on a barley based diet supplemented or not supplemented with a microbial β-glucanase.
Abbreviations: F, feed (without enzyme); SF, feed supplemented with the Clostridial enzyme; B, barley; Cr, crop content of a supplemented bird.

To test these possibilities, beta-glucanse activity of non-supplemented feed and of barley was determined using the qualitative Congo Red plate assay described above. The results demonstrate that the feed and barley have similar beta-glucanase activity, suggesting that feed endogenous beta-glucanses are from barley origin. Beta-glucanase activity was quantified in supplemented and non-supplemented feed using azo-barley glucan as the substrate. The data, presented in Figure 3.4 (Panel A), confirms that non-supplemented feed presents considerable levels of beta-glucanase activity although the quantified levels were 7,7 times lower than the ones observed for supplemented feed. In addition, crop beta-glucanase activity of non-supplemented birds was only 4 times lower when compared with the supplemented animals. Since Lic26-Cel5 is resistant to proteolytic inactivation, it is suggested that a fraction of the identified crop beta-glucanase activity has potentially a microbial origin (Figure 3.4, Panel B). As a result of the described endogenous beta-

glucanase activity, viscosity of the duodenal contents of both supplemented and non-supplemented birds was shown to be similar, as it is displayed in Figure 3.4 (Panel C). This is not completely unexpected and suggests that the residual endogenous beta-glucanase activity is sufficient to decrease the duodenal viscosity of non-supplemented birds to the levels observed in animals supplemented with the exogenous enzyme. One of the major demonstrated actions of feed beta-glucanases is to decrease the degree of polymerization of soluble glucans, by randomly cleaving glycosydic bonds in the xylan backbone (Bedford & Morgan, 1996; Fengler & Marquardt, 1988). Therefore, it is possible that the moderate levels of beta-glucanase activity observed in non-supplemented animals are responsible for decreasing the degree of polymerization of the soluble glucans to an extent that can contribute to significantly decrease the levels of digesta viscosity.

To analyse the potential changes in the molecular architecture of the endogenous and recombinant β-glucanases, during passage through the GI tract, digestive samples from birds supplemented and not supplemented with the exogenous enzyme were subjected to zymogram analysis. The data, displayed in Figure 3.5, demonstrated that Lic26-Cel5 is prone to proteolytic cleavage in the birds' GI tract, which occurs mainly in the crop and gizzard (Panel B and C). In Panel C it is shown that at the gizzard and in the subsequent digestive compartments, the 70 kDa Lic26-Cel5 is almost completely cleaved in the linker region connecting the Lic26 and the Cel5 modules, therefore releasing the two 32-35 kDa catalytic domains which still retain significant catalytic activity. However, as it has been suggested above, transformation of the bi-modular protein in two different enzymes has no impact in the catalytic efficiency of the microbial recombinant enzymes when hydrolysing barley β-glucan. Zymogram analysis of proteins from feed not subjected to exogenous cellulase supplementation confirms the presence of a 70-80 kDa enzyme with beta-glucanase activity in the cereal-based diet (Figure 3.5, Panel A). In addition, zymogram analysis of crop proteins from non-supplemented animals suggests the presence of a range of beta-glucanases with sizes ranging from 30 to 90 kDa (Figure 3.5, Panel B). The variety of enzymes displaying beta-glucanase activity identified in the crop of non-supplemented animals suggests a microbial origin.

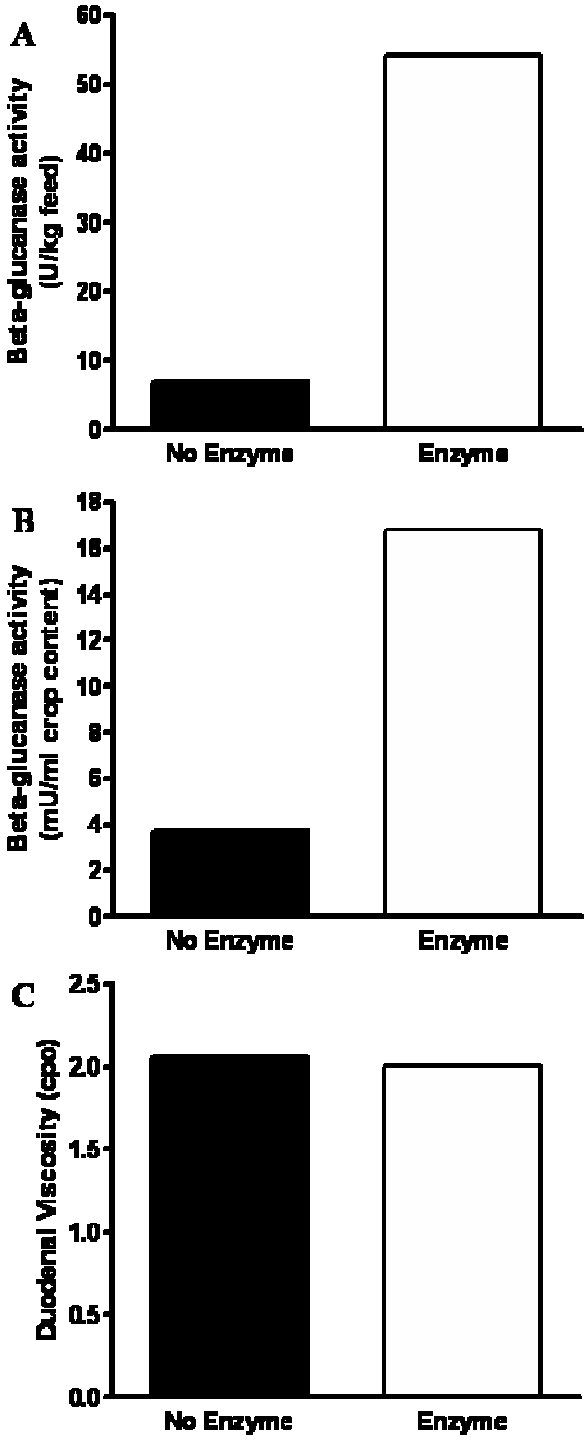

Figure 3.4 Viscosity and enzyme activities of barley-based feed and duodenal contents. In panel A, it is presented the beta-glucanase activity of the barley-based feed supplemented and not supplemented with the recombinant β-glucanase *Ct*Lic26A-Cel5E. The corresponding beta-glucanase activity in the crop contents of birds consuming the barley based feed supplemented with and without the recombinant enzyme is presented in Panel B. Viscosity of duodenal contents of birds consuming barley based feed with and without enzyme supplementation (Panel C).

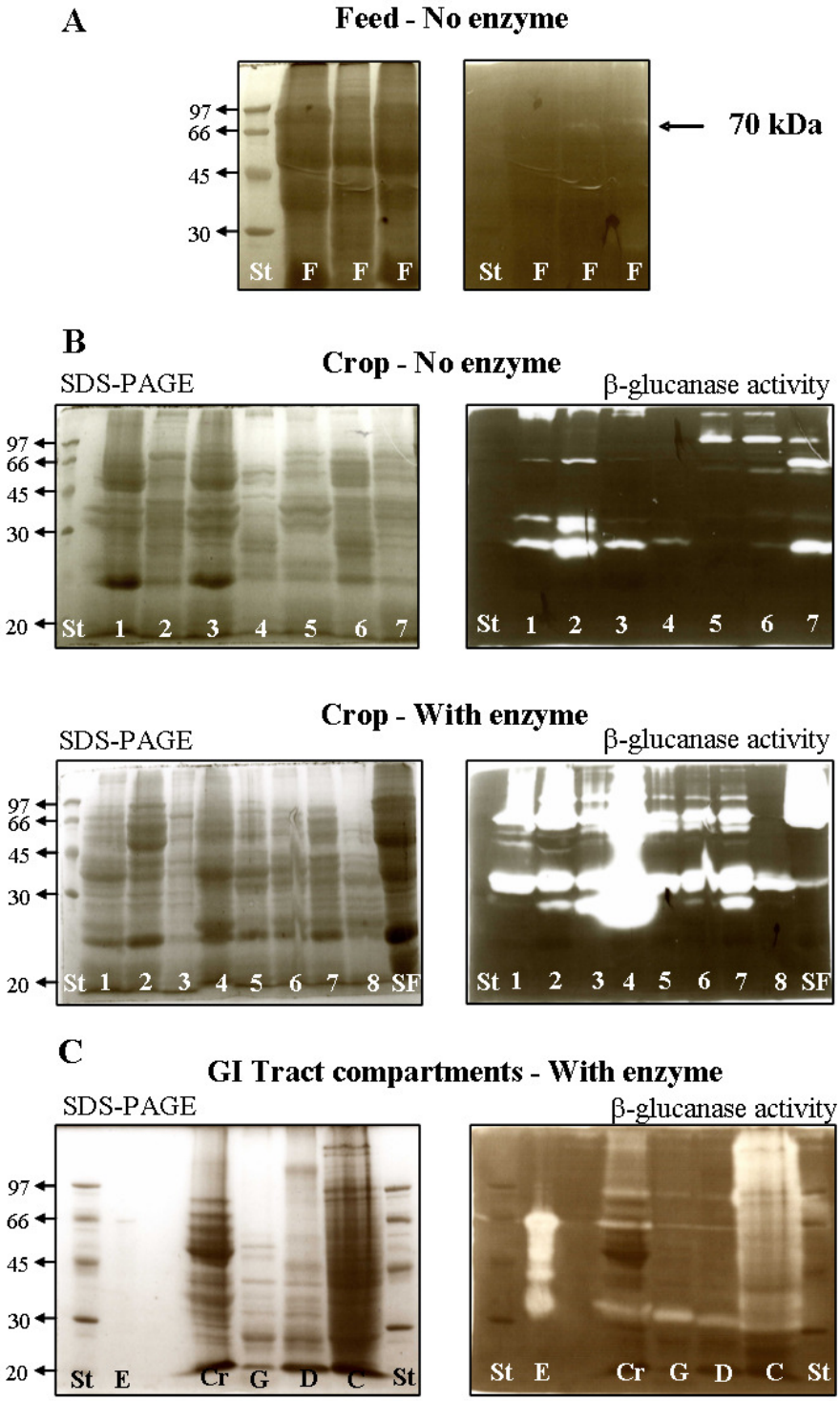

Figure 3.5 Zymogram analysis of not supplemented feed (Panel A), crop samples collected from birds supplemented or not with β-glucanase *Ct*Lic26A-Cel5E (Panel B), and digesta samples collected from various regions of the GI tract of birds supplemented with β-glucanase *Ct*Lic26A-Cel5E (Panel C).
Abbreviations: F, feed (without enzyme); St, low molecular weight protein standards; SF, supplemented feed; E, enzyme; Cr, crop; G, Gizzard; D, duodenum; C, Caecum.

Taken together, results presented here suggest that in free-range pastured broilers of a slow-growing genotype and at later stages of grow the presence of beta-glucanase activity expressed by crop microflora or from barley enzymes can contribute to depolymerise a significant proportion of the anti-nutritive beta-glucans characteristic of barley-based diets. Under these circumstances, the addition of a high dosage rate of a recombinant cellulase to the barley-based feed is not effective to improve bird performance possibly due to the presence of endogenous cellulase activity. If functional beta-glucanases are expressed by most barley varieties or are restricted to specific lots of this cereal remains to be established. In addition, it is possible that the crop beta-glucanase activity has both a plant and a microbial origin, which is only well established at later stages of the animals grow.

In conclusion the individual recombinant bi-modular cellulase from *C. thermocellum* is unable to improve the nutritive value of a barley-based feed available *ad libitum* for pastured broilers of a slow-growing genotype. The data suggests that although the enzyme suffers proteolysis on the GI tract it retains its full catalytic activity, suggesting that a lack of response to enzyme supplementation does not relate to enzyme degradation or inhibition. In contrast, non-supplemented animals present significant levels of beta-glucanases activity in the GI tract that is derived both from barley enzymes and, possibly, from proteins of microbial origin. Together the data suggest that the moderate levels of cellulase activity observed in the crop of non-supplemented animals are sufficient to degrade, partial or totally, the anti-nutritive beta-glucans present in barley based diets.

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CHAPTER 4 RESTRICTING THE INTAKE OF A CEREAL-BASED FEED IN FREE RANGE PASTURED POULTRY: EFFECTS ON PERFORMANCE AND MEAT QUALITY

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ABSTRACT

Pastures are assumed to be good sources of ALA and other bioactive compounds. In this study, the effects of restricting the intake of a cereal-based feed on the consumption of a leguminous-based pasture and, consequently, on poultry performance and meat quality were evaluated. Broilers of the RedBro Cou Nu × RedBro M genotype were fed on a cereal-based feed at different intake restriction levels (100%, 75% or 50% of the *ad libitum* intake), in portable floorless pens located on a subterranean clover (*Trifolium subterraneum)* pasture. Control birds were maintained at the same site in identical pens but had no access to pastures. The results revealed that, although growth rate achieved was below the levels expected for the genotype, restriction of the cereal based feed intake had a significant impact in broiler weight gain and feed conversion while leading to an increase of the relative leguminous pasture intake (from 1.6% to 4.9% of the total intake on a DM basis). In addition, bird performance was positively influenced by pasture consumption. The capacity of ingested pasture to modulate carcass characteristics, broiler meat fatty acid profiles and the meat content of total cholesterol, tocopherols and tocotrienols was investigated in broiler chicks slaughtered at day 64. Pasture intake decreased carcass yield ($P<0.05$) and meat pH ($P<0.001$) and improved breast skin pigmentation ($P<0.001$). The consumption of the leguminous pasture had a marginal effect in the vitamin E profiles and cholesterol contents of broiler meat ($P<0.05$), although it significantly affected meat fatty acid profile. Although pasture intake did not influence the contents of LA of poultry meat, the levels of n-3 PUFA in breast meat [ALA ($P<0.001$), EPA ($P<0.001$), DPA ($P<0.001$) and DHA ($P<0.001$)] were significantly higher in animals consuming leguminous biomass. Overall the data suggest an important deposition of ALA and some conversion of ALA to its derivatives in pastured broilers subjected to a cereal-based feed restriction.

***Key words**:* feed restriction, pasture intake, broiler performance, fatty acid profile.

4.1 INTRODUCTION

Current knowledge on the physiological functions of dietary PUFA, in particular n-3 PUFA, and the health benefits resulting from its regular consumption have led to an increased interest in the food sources of these nutrients (Sioen *et al*., 2006; Howe *et al*., 2006). Cardiovascular diseases, which are among the most important causes of human mortality in developed countries (Hu *et al*., 2001; Ganji *et al*., 2003), are highly related to the low ratios of P/S in Western diets. Moreover, PUFA contents of modern diets are low in n-3 fatty acids leading to high n-6/n-3 fatty acid ratios (Simopoulos, 2002). The imbalance in the n-6 *versus* n-3 proportion may be a contributing factor the pathogenesis of many diseases, including cancer, and inflammatory and autoimmune diseases (Simopoulos, 2004). In addition, it has been shown that consumption of long-chain n-3 fatty acids, such as EPA and DHA, which are vital components in the retina and the membrane phospholipids of brain cells, can positively affect various outcomes of cardiovascular disease (Rymer & Givens, 2005) and the incidence of metabolic syndrome (obesity, insulin resistance, or type 2 diabetes and dyslipidemia) (Nugent, 2004). By increasing the intakes of PUFA, particularly n-3 PUFA, the n-6/n-3 ratio can be reduced (Simopoulos, 2002).

Pasture may constitute a source of energy and protein for broilers raised under free range systems. In addition, the presence of a large range of bioactive compounds in the forage, such as xanthophylls and several hypocholesterolaemic and anticarcinogenic compounds, may lead to improvements in meat quality (Ponte *et al.*, 2004). Green forages are also a good source of tocopherols and tocotrienols, the natural diterpenes with vitamin E activity, which is the primary lipid-soluble antioxidant in biological systems (Kerry *et al*., 2000). Tocotrienols are also known to help lower plasma cholesterol levels (Qureshi *et al*., 1997). Antioxidant supplementation of feed is an efficient method for increasing meat oxidative stability (Maraschiello *et al*., 1999), although the various vitamin E forms are known to present different antioxidant potencies (Bourgeois, 1992). The contribution of green pasture vitamin E homologues for the oxidative stability of meat from chicken subjected to dietary restriction remains, however, to be established. Finally, meat provides from one third to one half of the daily-recommended cholesterol intake (300 mg; WHO, 2003), which seems to be directly associated with a greater risk of hypercholesterolemia

(Chizzolini et al., 1999). It has been previously shown that the inclusion of leguminous forages in broiler diets contributes to decreased cholesterol content of broiler meat (Ponte *et al.*, 2004). However, recently no changes were observed in cholesterol levels of broiler meat obtained when fresh (Ponte *et al.*, 2008b) or dehydrated forages (Ponte *et al.*, 2008c) were included in cereal-based diets for broiler chickens of both slow and fast growing genotypes.

Poultry meat has been considered as one of the main sources of PUFA for human diets, in particular n-3 PUFA (Sioen *et al*., 2006; Howe *et al*., 2006). It has been shown that the content of poultry meat in n-3 fatty acids, particularly in ALA, can be readily improved by increasing the levels of n-3 PUFA in poultry diets through the incorporation of linseed oil (López-Ferrer *et al*., 1999, 2001a) and/or oily fish by-products (Hulan *et al*., 1988; López-Ferrer *et al*., 2001b). However, a decrease in flavour quality has been reported for these products due to an overall higher meat susceptibility to lipid oxidation (Manilla & Husveth, 1999; Bou *et al*., 2001). It is well known that green pastures are a good source of ALA and pasture consumption leads, in ruminants, to higher contents of this fatty acid in meat while decreasing the n-6/n-3 fatty acid ratio (Wood & Enser, 1997; O'Sullivan *et al*., 2004). In a recent work developed in our laboratory, it was shown that pasture intake promotes the consumption of a cereal-based feed available for *ad libitum* consumption by free-range broilers, leading to an increased final body weight of broiler chicks of a slow growing genotype (Ponte *et al.*, 2008a). Although the levels of pasture intake were low, ranging between 2.5 to 4.5% of the total dry matter consumed, its effects on the fatty acid profile of broiler breast meat while marginal were significant, and resulted in increased EPA contents. In addition, lower levels of the n-3 precursor ALA were observed in meat from these animals, suggesting a higher conversion of ALA into EPA on birds consuming fresh forages (Ponte *et al.*, 2008b). In addition, broilers of a fast growing genotype grown under a conventional intensive system, with slaughtering at day 28 and fed on a diet containing 11% of a dehydrated leguminous-based forage, had meat with an improved fatty acid profile containing higher levels of n-3 long-chain (LC) PUFA (C≥20) (EPA, DPA and DHA) (Ponte *et al.*, 2008c). More recently, a study by Horsted and colleagues (2007) using laying hens feed organic diets with access to pasture, suggested that restricting the intake of a cereal-based feed promoted foraging and therefore consumption of the fresh forage. The objective of the research reported here was to establish the

effect of restricting the intake of a cereal-based feed on the consumption of a leguminous-based forage by free-range pastured broilers of a slow growing genotype. In addition, the implications of pasture intake on bird performance and the resulting meat contents of cholesterol, fatty acids, vitamin E homologues and β-carotene were evaluated.

4.2 MATERIAL AND METHODS

4.2.1 Animals, diets and management

This experiment was performed in the autumn of 2004 at Herdade dos Esquerdos (039º 07.18' north, 007º 29.36' west, 318 m a.s.l.), Vaiamonte, Portugal. During the experiment, the average daily mean temperature was 9.5 ºC (the mean of highest temperatures was 17.2 ºC and that of the minimum was 4.4 ºC) with seven days with rain and a total precipitation of 27 mm. Two hundred and forty 36-d-old males RedBro Cou Nu × RedBro M, vaccinated against Marek disease, were divided into 24 floorless portable metal outdoor pens (10 birds per pen), equalizing both the mean and the variance of body weight (BW). Previous to the commencement of the experiment animals were maintained in a conventional indoor facility following standard brooding procedures and fed on a typical maize soybean meal diet (see Figure 4.1). Animals were maintained in the pastured pens described below for an additional 28 days until slaughtered at day 64. The movable pens (1.7 m × 1.5 m × 0.5 m; 0.255 m^2 per bird) allowed birds to directly contact the legume-based pastures. Approximately one third of the top of each cage area was covered with transparent white-washed plastic for protection against harsh climatic conditions. Water was available for *ad libitum* consumption throughout the experiments and was provided via two automatic drinking nipples. A cereal-based feed was provided in one individual hanging tube feeder per pen. The composition of the cereal-based feed used in these studies, which was formulated to contain adequate nutrient levels as defined by the NRC (1994), is presented in Table 4.1.The birds were randomly assigned into one of the six treatments with 4 replicates of ten birds per treatment. The six treatments consisted of three levels of cereal-based feed consisting of 100% (100), 75% (75) or 50% (50) of the

reference intake to the breed and age (www.hubbard-isa.com), and two levels of pasture access, without access to pasture (No Pasture; Figure 4.2) or with access to a *Trifolium subterraneum* based pasture (Pasture; Figure 4.3), in a completely randomized experiment. To promote forage intake, the portable pens of the treatments with access to pasture were moved daily so that birds could consume of fresh herbage every day. Pens of the no pasture treatment were located in a fixed position, in the same field, and access to the pasture was blocked, in the initial days and throughout the experiment, by adding new pine wood shavings to the ground. At day 50, samples of the pasture were collected from 1 m^2 paddocks, by cutting it at 3 cm above the ground, for chemical evaluation. The experimental protocol was approved by the ethics commission of Centro Interdisciplinar em Investigação em Sanidade Animal (CIISA) following the appropriated European Union guidelines (N. 86/609/EEC).

Figure 4.1 Experiment animals maintained in a conventional indoor facility following standard brooding procedures prior to the beginning of the experiment.

Individual body weights were recorded weekly. 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, four birds per cage were killed by the intravenous injection of 2.5 ml of an aqueous solution containing 125 mg Tiopental Brown (Braun, Barcelona, Spain) in the wing vein

and crop content was collected. Forage particles were separated from cereal-based feed particles. The dry matter weight of the forage and the cereal-based feed fractions found in crops were measured. This allowed for the estimation of pasture consumption considering the intake of cereal-based feed and the dry matter content of the pasture and of the cereal-based feed. In addition, at day 64, three birds per pen were slaughtered at a commercial processing plant. The carcasses, obtained after defeathering, eviscerating and removing head, neck and extremities, were refrigerated for 24 hours and weighed. Meat pH was measured as described by Sierra (1973). After carcass measurements, skinless breast meat samples (approximately 10 g) were collected for determining total lipids, fatty acid composition, total cholesterol and vitamin E compounds, ground using a 750 W potency food processor (3 × 5 s), vacuum packed and stored at -80 ºC until required.

Figure 4.2 Aspect of birds without access to pasture

Figure 4.3 Aspect of the experimental field displaying birds with access to a *Trifolium subterraneum* based pasture

Table 4.1 Ingredient composition and calculated analysis of the cereal-based feed.

Ingredients	**%**
Corn	44.1
Wheat	26.0
Soybean meal 47%	27.0
Sodium chloride	0.30
Calcium carbonate	0.78
Dicalcium phosphate	0.98
Choline 75%	0.06
DL-Methionine	0.18
Mineral and vitamin premix 1	0.60
Calculated nutrient content	
Energy (kcal ME/kg DM)	2890
Crude protein (%)	19.1
Ether extract (%)	2.50
Crude fibre (%)	5.00
Ash (%)	6.00
Methionine (%)	0.42

[1] Mineral-vitamin premix provided the following per kg 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.

4.2.2 Skin colour

The colour of breast skin was evaluated using a Minolta chroma meter CR-300. The readings were taken on equivalent positions of the carcasses. The tip of the chromameter-measuring head was placed flat against the surface of the skin. For each reading 3 measurements were performed and the final value for each animal is the average of those readings. Skin colour was expressed in the CIELAB dimensions of lightness (L*), redness (a*) and yellowness (b*).

4.2.3 Determination of total lipids

Meat samples were lyophilized (-60 ºC and 2.0 hPa) to constant weight using a lyophilizer Edwards Modulyo (Edwards High Vaccum International, UK), maintained desiccated at room temperature and analyzed within two weeks. For total lipid determination, intramuscular fat was extracted as described by Alfaia *et al*. (2006) from the lyophilized samples (0.25 g). Total lipids were measured gravimetrically, in duplicate, by weighing the fatty residue obtained after solvent evaporation.

4.2.4 Determination of fatty acid composition

Intramuscular fat of lyophilized samples (0.25 g), cereal-based feed or pasture (0.10 g of dry matter) was firstly dissolved in 1 ml of dry toluene. Then, fatty acids were converted to methyl esters (FAME) by base-catalyzed transesterification with sodium methoxide for 2 h at 30 ºC. The fatty acid composition was determined by gas chromatography of FAME, performed with a Varian 3800 gas chromatograph (Varian Inc, Walnut Creek, CA, USA) equipped with a flame ionization detector and an OmegaWax 250 (Supelco, Bellefont, CA, USA) capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness). The chromatographic conditions were as follows: injector temperature, 250 ºC; detector temperature, 280 ºC; helium was used as carrier gas and the split ratio was 1:20. The gas chromatograph oven temperature was programmed to start at 150 ºC (maintained for 15 min) followed by a 3 ºC/min ramp to 220 ºC (maintained for 20 min). Identification

was accomplished by comparing the retention times of peaks from samples with those of FAME standard mixtures. Quantification of FAME was based on the internal standard technique, using nonadecanoic acid (19:0) as internal standard (NU-Chek-Prep. Inc.,Elysian, MN, USA) and on the conversion to relative peak areas to weight %, using the corrected response factor of each fatty acid (ES ISO 5508, 1990). Fatty acids were expressed as gravimetric contents (mg/g muscle) or as a percentage of the sum of identified fatty acids (% w/w).

4.2.5 Quantification of total cholesterol, tocopherols and tocotrienols

The simultaneous determination of total cholesterol, β-carotene, tocopherols and tocotrienols was performed as described by Prates *et al*. (2006). The method involves a direct saponification of the fresh meat (0.75 g), cereal-based feed or pasture (0.10 g of dry matter), a single *n*-hexane extraction and the analysis of the extracted compounds by normal-phase HPLC, using fluorescence (tocopherols and tocotrienols) and UV-Vis photodiode array (cholesterol and β-carotene) detections in tandem. Briefly, the analysis were performed using a normal-phase silica column (Zorbax RX-Sil with the corresponding 12.5 mm analytical guard column, 4.6 mm ID 250 mm, 5 μm particle size, Agilent Technologies Inc., Palo Alto, CA, USA), with fluorescence detection for tocopherols (excitation wavelength of 295 nm and emission wavelength of 325 nm) and UV–Vis photodiode array detection for cholesterol (202 nm) and β-carotene (450 nm) in series. The solvent (1% v/v isopropanol in *n*-hexane) flow rate was 1 ml/min, the run last for 17 min and the temperature of the column oven was adjusted at +20º C. The injection volumes used varied between 20 and 100ul in order to get values within the linearity range of the standard curves. The contents of total cholesterol, β-carotene, 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.

4.2.6 Statistical analysis

Statistical analysis was conducted by analysis of variance using SAS with the GLM procedure (SAS Institute, 2004). The model used for analysing data of the pasture experiment included the effect of pasture intake (P), the effect of cereal-based feed restriction (R) and the interaction between P and R (P×R). The experimental unit considered was the pen. The significance for main effects of pasture, restriction and the interaction between these two effects were presented. Orthogonal contrasts were constructed for testing differences between restriction levels. The first contrast (100 *vs.* 75) compared the parameters from birds with no restriction (100) with animals subjected to a 75% of the referenced intake (75). The second contrast (75 *vs.* 50) compared the parameters from birds with 75% restriction (75) with animals with access to 50% of its referenced intake (50). Unless otherwise stated, differences were considered significant when $P<0.05$.

4.3 RESULTS AND DISCUSSION

4.3.1 Leguminous-based pasture and cereal-based feed composition

Total fatty acids were higher in the cereal-based feed than in pasture (Table 4.2). As expected, LA was the major fatty acid of the cereal-based diet, while ALA predominated in the leguminous-based pasture. Palmitic acid (16:0) is relatively abundant in both feeds. The cereal-based feed contained higher percentages of oleic acid (18:1n-9) when compared with the pasture. In addition, EPA and DHA were present at very low levels (data not shown). The ratio of LA/ALA was 0.15 and 16.7 in pasture and in cereal-based feed, respectively.

Although γ-tocopherol was co-eluted with a minor proportion of β-tocotrienol a profile of vitamin E compounds was obtained (Table 4.2). The α- and γ-tocopherols are the most abundant vitamin E homologues both in the cereal-based diet and leguminous-based

pasture. In addition, when compared with the cereal-based feed, pasture biomass had significant levels of β-carotene.

Table 4.2 Total fatty acids (mg g-1 dry matter), fatty acid composition (% w/w), diterpenes (tocopherols and tocotrienols) and β-carotene (μg g-1 dry matter) of the cereal-based feed and of the T. subterraneum based pasture used in the experiment.

	Cereal-based feed[1]	**Pasture**
Total fatty acids	19.89	4.51
Fatty acids		
14:0	0.08	0.28
16:0	14.03	13.80
16:1n-7	0.19	0.12
17:0	0.15	0.11
18:0	2.64	1.62
18:1n-9	21.60	1.90
18:2n-6	57.85	10.65
18:3n-3	3.46	71.52
Diterpenes		
α-Tocopherol	36.9	69.3
α-Tocotrienol	6.48	5.62
β-Tocopherol	2.17	0.78
γ-Tocopherol[2]	20.5	11.2
γ- Tocotrienol	3.60	0.80
δ-Tocopherol	1.49	3.91
β-Carotene	nd[3]	66.3

[1] The cereal-based feed was supplemented with α-tocopherol (20 mg kg^{-1}); [2] Co-eluted with a small proportion of β-tocotrienol; [3] nd, not detected

4.3.2 Pasture intake and bird performance

Although pasture consumption was low, cereal-based feed restriction resulted in a significantly higher ($P<0.05$) proportion of pasture intake in the total intake (PI/TI;Table 4.3). Leguminous-based pasture was estimated to constitute 1.6 %, 2.8% and 4.9% (DM basis) of the total intake in animals consuming the cereal-based feed at 100%, 75% and 50% of the reference intake, respectively. Analogous results were obtained in a study with

organic laying hens (Horsted *et al.*, 2007). In addition, the recorded percentages of forage intake in this study are lower than those reported by Ponte *et al*. (2008a), in experiments with *ad libitum* supply of cereal-based feed. However, these values should be viewed with some caution, since they represent an estimate of the pasture consumption at a specific moment of the trial and forage consumption may have varied during the experiment. It has been shown that climatic factors and time of the day have important effect on range area used and forage consumption in laying hens (Hegelund *et al.*, 2005; Horsted *et al.*, 2007). Interestingly, although the proportion of pasture intake increased with the level of restriction, the absolute pasture consumption was equivalent in all groups. Total pasture intake, estimated according to the cereal-based feed intake, varied between 400g to 474g (DM basis) during the 28 days of the experiment. The mortality rate during this experiment was moderate (5.6%) and was not related with the treatments (data not shown).

Figure 4.4 Aspect of chickens on movable pens on a leguminous based pasture

As expected, the imposed restriction on cereal-based feed intake had a significant effect (P<0.001) both on weight gain and on body weight (Table 4.4). Animals of treatments 75 and 50 displayed lower weight gains and body weights when compared with animals from the non-restricted groups. Although no significant difference was observed in the weight gains as a result of the cereal-based feed intake restriction in the last week of the experiment (P>0.05), total weight gain and final body weights were significantly decreased in animals of the cereal-based feed restricted groups (P<0.001). Other researchers have also observed that limiting feed intake depresses growth during the period of restriction (Acar *et al.*, 1995; Govaerts *et al.*, 2000). Birds subjected to the most severe feed restriction were not able to compensate the reduction in performance resulting from the reduction of cereal-based feed intake with an increase in pasture intake and, therefore, a significant decrease on growth rate was observed. As expected, the data suggest that the high fibre content of pasture biomass limits its feed intake and also nutrient utilization. Nevertheless, although no differences were observed between the weight gain and body weight in birds consuming and not consuming pasture throughout the trial, a cumulative effect was observed that allowed the total weight gain (P<0.01) and, consequently, the final body weight (P=0.075) to be higher in grazing animals.

The data suggested that feed conversion ratio (computed considering only the cereal-based feed intake) was only influenced by feed restriction when this was imposed at its highest rate (P>0.05). Feed conversion efficiency of animals from treatment 50 was lower when compared with birds originated on other treatments. Bowes *et al*. (1988) observed that FCR was higher in broilers restricted in feed intake by 25% when compared with the *ad libitum* fed group. The restriction reduced the intake of nutrients and metabolizable energy and, consequently, birds grew significantly less, therefore expending a higher proportion of the dietary energy and nutrients in maintenance. This negative effect on FCR would have been worse if the restricted birds were fed until they achieved the final weight of non restricted animals. Feed conversion ratios observed in animals consuming pasture was lower than in animals without access to the leguminous-based pasture (P<0.05). Similar improvements in weight gain and final body weight were obtained in a previous study, although no significant change was observed in feed conversion ratio of pastured poultry with *ad libitum* access to cereal-based feed (Ponte *et al.*, 2008a). Finally, data presented here suggest that the growth rate achieved by the broilers, is below the levels

expected for the genotype RedBro Cou Nu x RedBro M (2412 g of BW at day 63 – Hubbard ISA management manual), even when cereal-based feed was available at 100% of the referenced feed intake. Moreover, feed conversion ratios were considerably higher than expected for this genotype (should be 2.29-2.35 at day 63), suggesting that, considering the suboptimal environmental conditions to which the free- range chicken were subjected when compared with birds housed indoors, animals used the consumed energy to compensate inappropriate temperatures.

Table 4.3 Intake of free-range broilers fed on a cereal-based feed, supplying 100% (100), 75% (75) or 50% (50) of the referenced intake, foraging in *Trifolium subterraneum* based pasture.

	Pasture			SEM	Significance level		
					R	Contrasts	
	100	75	50			100 *vs.* 75	75 *vs.* 50
PI/TI (%)							
Dry matter basis	1.59	2.75	4.91	0.770	*	ns	ns
Fresh matter basis	8.68	12.1	18.6	2.707	+	ns	ns
Intake (g)							
Cereal based feed (actual)	4134	3101	2067	-	-	-	-
Pasture (estimated)	399.9	448.9	474.3	114.72	ns	ns	ns

Significance: ns, $P>0.05$; * $P<0.05$; + $P<0.10$; SEM, standard error of mean. The symbols used mean as follow: PI/TI, pasture intake/total intake; R, feed restriction.

Table 4.4 Performance of free-range broilers fed on a cereal-based feed, supplying 100% (100), 75% (75) or 50% (50) of the referenced intake, without access to pasture or foraging in *Trifolium subterraneum* based pasture.

	No Pasture			Pasture			SEM	Significance level				
								P	R	PxR	Contrasts	
	100	75	50	100	75	50					100 *vs.* 75	75 *vs.* 50
BW (g)												
36d	971	985	976	962	937	964	21.2	ns	ns	ns	ns	ns
42d	1193	1167	1048	1194	1123	1068	20.8	ns	***	ns	*	***
50d	1509	1348	1132	1531	1316	1162	24.5	ns	***	ns	***	***
56d	1808	1536	1234	1843	1544	1293	30.3	ns	***	ns	***	***
64d	2046	1767	1410	2103	1800	1498	38.3	ns	***	ns	***	***
WG (g)												
36-42d	222	182	72	232	187	103	15.0	ns	***	ns	**	***
42-50d	316	182	84	337	192	94	13.9	ns	***	ns	***	***
50-56d	299	188	102	313	229	131	29.6	ns	***	ns	**	**
56-64d	238	231	176	259	256	204	27.4	ns	ns	ns	ns	ns
36-64d	1075	783	434	1140	863	533	35.2	**	***	ns	***	***
FCR[1]												
36-64d	3.85	3.96	4.87	3.65	3.59	3.98	0.252	*	*	ns	ns	*

Significance: ns, P>0.05; *, P<0.05; ** P<0.01; *** P<0.001; + P<0.10; Means with the same column bearing different superscripts are significantly different (P<0.05); SEM, standard error of mean. The symbols used mean as follow: BW, body weight; WG, weight gain; FCR, feed conversion ratio; R, feed restriction; P, pasture, PxR, interaction between pasture intake and feed restriction; [1] Feed conversion are relative to the cereal-based feed.

4.3.3 Meat physical properties

Both restriction of feed intake and pasture consumption had a negative effect on carcass yield ($P<0.001$ and $P<0.05$, respectively; Table 4.5). In addition, a significant interaction between pasture intake and feed intake restriction ($P<0.01$) resulted in a higher decrease on carcass yield in birds consuming pasture when compared with birds without access to pasture, which were subjected to the feed intake restriction. It is widely accepted that dietary fibre can influence the development and the size of digestive organs in broilers chicks (Brenes *et al.*, 1993; Mourão *et al*. 2008), which in turn has a significant influence in carcass yield. It is also known that birds with lower live weights due to a lower intake of nutrients, like that ones subjected to a feed restriction, usually have a lower carcass yield (Havenstein *et al.* 2003). In addition, higher activity of grazing animals in conjunction with the imposed feed restriction might have improved the proportion of wings, thighs and drum sticks (Castellini *et al.*, 2002). In contrast, Fanatico *et al*. (2005) found no differences in the carcass yield of indoor and outdoor birds and increases in carcass yields were observed in pastured broilers (Ponte *et al.*, 2008a). In addition, decreases in carcass yield in birds with access to pasture suggest that foraging could increase the proportion of GI tract tissues on the overall BW, as an adaptation to a higher fibre intake. Meat pH was lower in breast originated from grazing animals ($P<0.001$). This result may reflect the different management conditions of pasture animals that are allowed grazing, therefore allowing more activity (Castellini *et al*., 2002; Fanatico *et al*., 2007). The significant interaction between the effect of feed restriction and pasture intake ($P<0.05$) resulted in a greater decrease on meat pH in birds consuming pasture when compared with birds without access to pasture that were subjected to feed restriction.

Table 4.5 Carcass yield, breast meat pH and skin colour of free-range broilers fed on a cereal-based feed, supplying 100% (100), 75% (75) or 50% (50) of the referenced intake, without access to pasture or foraging in *Trifolium subterraneum* based pasture.

	No Pasture			Pasture			SEM	Significance level				
								P	R	PxR	Contrasts	
	100	75	50	100	75	50					100 *vs.* 75	75 *vs.* 50
Carcass yield (%)	63.12^{ab}	61.90^{b}	62.82^{ab}	64.72^{a}	60.91^{b}	58.03^{c}	0.891	*	***	**	**	ns
Meat pH	5.66^{ab}	5.66^{ab}	5.75^{a}	5.62^{b}	5.54b^{cd}	5.49^{d}	0.043	***	ns	*	ns	ns
Skin colour												
…..L*	57.9	59	59.9	56.8	58.4	59.4	0.982	ns	*	ns	ns	ns
…..a*	1.98	1.51	1.39	2.03	0.79	0.97	0.339	ns	*	ns	**	ns
…..b*	4.94^{b}	2.86^{b}	3.98^{b}	4.90^{b}	8.42^{a}	10.77^{a}	0.968	***	*	***	ns	ns

Significance: ns, P>0.05; *, P<0.05; ** P<0.01; *** P<0.001; Means with the same column bearing different superscripts are significantly different (P<0.05); SEM, standard error of mean. The symbols used mean as follow: NR, no feed restriction; R, feed restriction; P, pasture, PxR, interaction between pasture intake and feed restriction; L*, lightness; a*, redness b*, yellowness.

Results of the colorimetric evaluation of breast skin, presented as the CIELAB values of L (lightness), a (redness) and b (yellowness), revealed that birds with 75% or 50% of the referenced feed intake displayed higher L scores ($P<0.05$), indicating a less deeply pigmented skin (Table 4.5). Interestingly, feed restriction induced a considerable decrease ($P<0.01$) in the broiler carcass redness (a), showing that the usually undesirable pink and red tones in the skin were less developed. Pasture intake did not influence broiler skin L and a values ($P>0.05$). Breast skin yellowness (b) was influenced by feed restriction ($P<0.05$), pasture intake ($P<0.001$) as well as by the interaction between this two effects ($P<0.001$). In animals consuming leguminous-based pasture, feed restriction had the opposite effect, inducing a large increase in breast skin yellow tones, most probably supplied by the natural pigments present in the leguminous-based pasture. Nevertheless, no differences were observed in the breast skin b scores between animals without feed restriction as a result of pasture consumption. This is supported by the observation that although pasture contains carotenoid pigments (Toyopmizu *et al.*, 2001 and Table 4.2), no improvement in breast skin yellowness is observed when diets contain a considerable proportion of corn (Schaible, 1970).

4.3.4 Fatty Acid Composition, Cholesterol, Tocopherols and Tocotrienols of Meat

The predominant fatty acids in meat from birds of all treatments were palmitic (16:0) and stearic (18:0) acids as SFA, oleic acid as MUFA, and LA and arachidonic acid (20:4n-6) as PUFA (Table 4.6). Oleic and palmitic acids were the most abundant fatty acids in the various meats under analysis. The restriction on the intake of the cereal-based feed induced several changes in the fatty acid profile of broiler meat. The percentages of 18:1n-9 and 22:2n-6 fatty acids were lower in the meat from birds receiving only the cereal-based feed at 75 or 50% of the referenced intake. However, the restriction on the cereal-based intake had an opposite effect in the levels of the 17:0, 18:0, 20:1n-9, 20:4n-6, 20:5n-3, 22:5n-3 and 22:6n-3. Together the data suggest that the significant increase in stearic acid and decrease in oleic acid results from a depression in the activity of the stearoyl-CoA desaturase (SCD) in animals of the forage consuming group. It is well known that the

SCD expression may be regulated by nutritional factors (Rosebrough *et al.*, 2005). In addition, pasture consumption influenced the fatty acid profile of broiler meats, leading to significant increases in most PUFA. Although no changes were observed in the LA and 18:3n-6 contents of meat from birds with access to pasture, all the other n-6 PUFA levels were enhanced ($P<0.05$). However, a significant effect of pasture intake in fatty acid content was observed for all the n-3 PUFA percentages ($P<0.001$). The levels of ALA was influenced by pasture intake ($P<0.001$). However ALA contents were affected differently by pasture intake as analysed by birds subjected to the different restriction levels ($P<0.001$). In animals without access to pasture, feed restriction resulted in lower ALA contents in meat. In contrast, in animals consuming the leguminous-based pasture, feed intake restriction had the opposite effect, inducing a large deposition of the main n-3 PUFA in breast meat, which were most possibly supplied by the leguminous-based pasture in which ALA is the most abundant fatty acid. Levels of LC n-3 PUFA, EPA, DPA and DHA ($P<0.001$), were higher in birds consuming pasture. Nevertheless, since LC n-3 PUFA were present in trace levels in both the cereal-based feed and in pasture, these data suggest a significant conversion of ALA to its derivatives in broilers with access to pasture, by desaturation and elongation. Similar results have been obtained in previous studies in birds consuming linseed oil (López-Ferrer *et al.*, 2001a) and leguminous biomass (Ponte *et al.*, 2008b, Ponte *et al.*, 2008c; Mourão *et al.*, 2008). This finding supports the ability of chicken to deposit ALA in muscle tissues and to convert this FA to its derivatives. However, the levels of ALA, EPA, DPA (22:5n-3), and DHA in the pasture consuming broiler meat are much lower when compared with the percentages of the n-3 fatty acids reported in meat originated in birds supplemented with 2-4% of fish oil (López-Ferrer *et al.*, 2001a). This may be explained, at least in part, by the fact that ALA present in pasture is in the esterified form in structural lipids, including galactolipids from chloroplasts (Gurr, 1984). Thus, the broiler digestive system may not be able to generate free ALA via hydrolysis of galactolipids due to a lack of galactolipase activity.

Table 4.6 Fatty acid composition (% w/w) in breast meat of free-range broilers fed on a cereal-based feed, supplying 100% (100), 75% (75) or 50% (50) of the referenced intake, without access to pasture or foraging in *Trifolium subterraneum* based pasture.

	No Pasture			Pasture			SEM	Significance level				
								P	R	PxR	Contrasts	
	100	75	50	100	75	50					100 *vs.* 75	75 *vs.* 50
14:0	0.43^{ab}	0.40^{bc}	0.41^{bc}	0.48^{ab}	0.51^{a}	0.33^{c}	0.036	ns	*	*	ns	*
15:0	0.26	0.32	0.33	0.32	0.30	0.34	0.018	ns	ns	ns	ns	ns
16:0	24.6	23.8	23.3	22.1	24.1	22.4	0.912	ns	ns	ns	ns	ns
16:1n-7	2.28	2.16	2.03	2.19	2.25	1.84	0.147	ns	ns	ns	ns	ns
17:0	0.13	0.13	0.15	0.13	0.14	0.14	0.005	ns	***	ns	ns	**
18:0	12.5	13.0	13.6	12.9	12.7	13.7	0.591	ns	**	ns	ns	**
18:1n-9	26.1	24.4	22.4	26.8	23.9	20.6	0.735	ns	***	ns	**	***
18:2n-6	19.7	20.2	20.2	18.5	19.71	20.6	0.441	ns	*	ns	ns	ns
20:0	0.10^{b}	0.10^{b}	0.12^{a}	0.10^{b}	0.10^{b}	0.10^{b}	0.003	ns	***	*	ns	**
18:3n-6	0.09	0.08	0.07	0.08	0.08	0.08	0.004	ns	ns	ns	ns	ns
20:1n-9	0.25	0.24	0.22	0.25	0.22	0.20	0.009	ns	***	ns	*	*
18:3n-3	0.45^{bc}	0.41^{cd}	0.38^{d}	0.42^{cd}	0.50^{ab}	0.54^{a}	0.024	***	ns	***	ns	ns
20:2n-6	0.34	0.35	0.38	0.41	0.39	0.38	0.023	*	ns	ns	ns	ns
20:3n-6	1.05	1.08	1.09	1.19	1.2	1.22	0.053	**	ns	ns	ns	ns
20:4n-6	7.99	9.27	10.6	9.47	9.15	12.0	0.529	**	***	ns	ns	***
20:3n-3	0.02	0.02	0.02	0.03	0.04	0.04	0.002	***	ns	ns	ns	ns
20:5n-3	0.19	0.22	0.24	0.25	0.30	0.36	0.017	***	***	ns	*	*
22:2n-6	0.06	0.05	0.05	0.09	0.07	0.05	0.008	*	**	ns	ns	ns
22:4n-6	1.60	1.66	1.90	1.85	1.76	2.06	0.089	*	**	ns	ns	**
22:5n-3	0.81	0.90	0.98	1.03	1.09	1.30	0.052	***	***	ns	ns	**
22:6n-3	1.09	1.27	1.47	1.52	1.49	1.73	0.093	***	**	ns	ns	*

Significance: ns, $P>0.05$; *, $P<0.05$; ** $P<0.01$; *** $P<0.001$; Means with the same column bearing different superscripts are significantly different ($P<0.05$); SEM, standard error of mean. The symbols used mean as follow: R, feed restriction; P, pasture, PxR, interaction between pasture intake and feed restriction.

Confirming the tendency of individual fatty acids presented in Table 4.6, the restriction in cereal-based feed intake contributed to increase total PUFA, n-3 PUFA and n-6 PUFA content of broiler meat ($P<0.001$), induced by a decrease in the content of MUFA, particularly of oleic acid (Table 4.7). Although pasture consumption did not result in significant changes in total n-6 PUFA, n-3 PUFA percentages were improved ($P<0.001$). Consequently, the n-6/n-3 ratio in meat derived from grazing birds was lower than in animals consuming exclusively the cereal-based feed. Interestingly, the effect of pasture intake was evident even in birds consuming the cereal-based feed at the level of 100% of the referenced intake. This observation is surprising since it contradicts previous observations that suggested that at a non-restricted level of intake of the cereal-based feed, pasture intake had no effect on the n-6/n-3 ratios of boiler breast meat (Ponte *et al*., 2008b). Reference intakes stated in breed standards consider typical housing facilities. However, it is well known that free-range production systems are usually associated with higher feed intakes that allow birds to counteract harsh weather conditions. Therefore, it is likely that under the present experiment birds of the 100% intake treatment could have been subjected to a slight consumption restriction, which could have increased pasture intake. This is supported by the observation that pasture intake (Table 4.3) was remarkably similar across the three restriction treatments in birds of the grazing treatments. Taken together, the data suggest that free access to high-quality pastures by free range pastured broilers subjected to a minor intake restriction of the cereal-based feed, might be sufficient to improve the n-3 fatty acid content of broiler meat.

Meat glycerol lipids (or non-sterol lipids) were decreased by imposing the feed restriction in free-range broilers ($P<0.001$), although their contents were not affected as a result of pasture consumption ($P>0.05$; Table 4.7). However, all chicken meats are lean, based on the Food Advisory Committee (1990) criteria (<5% fat), and depicted median contents of total cholesterol (0.42-0.46 mg/g), when compared with those reviewed by Chizzolini *et al.* (1999) for beef. Meat total cholesterol was increased in meat from animals subject to the highest feed restriction and in animals with access to the pasture ($P<0.05$). However, the increase in meat cholesterol concentration as influenced by the level of feed restriction was exclusive to the animals without access to the pasture ($P<0.05$).

Table 4.7 Total lipids and cholesterol contents (mg/g meat), selected sums of fatty acids (%w/w) and nutritional ratios in breast meat of free-range broilers fed on a cereal-based feed, supplying 100% (100), 75% (75) or 50% (50) of the referenced intake, without access to pasture or foraging in *Trifolium subterraneum* based pasture.

	No Pasture			Pasture			SEM	Significance level				
								P	R	PxR	Contrasts	
	100	75	50	100	75	50					100 *vs.* 75	75 *vs.* 50
Total lipids	9.15	7.77	7.45	8.57	7.18	7.20	0.380	ns	***	ns	***	ns
Cholesterol	0.42^{c}	0.43^{bc}	0.46^{a}	0.46^{a}	0.44^{ab}	0.45^{a}	0.007	*	ns	*	ns	*
Partial sums												
SFA	38.0	37.8	37.9	36.0	37.8	37.0	0.724	ns	ns	ns	ns	ns
MUFA	28.6	26.8	24.7	29.2	26.4	22.7	0.850	ns	***	ns	ns	ns
PUFA	33.4	35.4	37.4	34.8	35.8	40.3	1.009	ns	***	ns	ns	**
n-3	2.56	2.82	3.08	3.26	3.42	3.97	0.133	***	***	ns	ns	**
n-6	30.8	32.6	34.3	31.6	32.4	36.3	0.915	ns	***	ns	ns	**
Ratios												
P/S	0.88	0.94	0.99	1.0	0.95	1.1	0.061	ns	ns	ns	ns	ns
n-6/n-3	12.0	11.6	11.2	9.75	9.57	9.31	0.276	**	0.073	ns	ns	ns

Significance: ns, $P>0.05$; *, $P<0.05$; ** $P<0.01$; *** $P<0.001$; + $P<0.10$; Means with the same column bearing different superscripts are significantly different ($P<0.05$); SEM, standard error of mean. The symbols used mean as follow: R, feed restriction; P, pasture, PxR, interaction between pasture intake and feed restriction.

Table 4.8 Diterpenes (tocopherols and tocotrienols) and β-carotene contents (μg/g meat) in chicken breast meat of broilers fed on a cereal-based feed, supplying 100% (100), 75% (75) or 50% (50) of the referenced intake, without access to pasture or foraging in *Trifolium subterraneum* based pasture.

	No Pasture			Pasture			SEM	Significance level				
								P	R	PxR	Contrasts	
	100	75	50	100	75	50					100 *vs.* 75	75 *vs.* 50
Diterpenes												
α-Tocopherol	3.09	3.13	3.15	2.65	2.57	2.82	0.192	**	ns	ns	ns	ns
α-Tocotrienol	0.68	0.76	0.63	0.66	0.62	0.65	0.056	ns	ns	ns	ns	ns
β-Tocopherol	0.06	0.07	0.06	0.06	0.05	0.05	0.005	*	ns	ns	ns	ns
γ-Tocopherol[1]	0.56	0.53	0.54	0.49	0.44	0.47	0.029	**	ns	ns	ns	ns
γ-Tocotrienol	1.16	1.54	1.1	1.26	1.13	1.29	0.151	ns	ns	ns	ns	ns
δ-Tocotrienol	0.13	0.17	0.12	0.14	0.11	0.14	0.017	ns	ns	ns	ns	ns
β-Carotene	0.013	0.016	0.017	0.018	0.017	0.02	0.002	ns	ns	ns	ns	ns

Significance: ns, $P>0.05$; *, $P<0.05$; ** $P<0.01$; Means with the same column bearing different superscripts are significantly different ($P<0.05$); SEM, standard error of mean. The symbols used mean as follow: R, feed restriction; P, pasture, PxR, interaction between pasture intake and feed restriction; 1 Co-eluted with small amounts of β-tocotrienol.

The diterpenes α-tocopherol and γ-tocotrienol were the major vitamin E homologues detected in broiler breast meat (Table 4.8). In addition, breast meat presented trace levels of α-tocotrienol, β-tocopherol and γ-tocopherol (the latest co-eluted with a minor proportion of β-tocotrienol). The prevalence of α-tocopherol in meat is well established and results from the more than tenfold preference of the tocopherol-binding protein for α-tocopherol in relation to the γ-homologues, which are the most common vitamin E molecules in plant foods (Decker *et al.*, 2000). Restriction of cereal-based feed intake had no effect on diterpene deposition in broiler meat. Meat from birds with access to the leguminous-based pasture presented lower levels of α-tocopherol ($P<0.01$), β-tocopherol ($P<0.05$) and γ-tocopherol ($P<0.01$) (Table 4.8). This is not completely unexpected since the cereal-based feed displayed higher concentrations of these vitamin E homologues (Table 4.2). In addition, although the leguminous based forage had significant levels of β-carotene, pasture intake had no influence on the levels of this lipid-soluble antioxidant pro-vitamin in breast meat.

In conclusion, in this study the imposed restriction on the intake of a cereal-based feed for free-range broilers led to a higher proportion of pasture intake in the overall total intake. Although feed restriction had a detrimental effect on broiler performance and growth rate achieved by the broilers was below the levels expected for the genotype, pasture consumption had the capacity to, at least partially, counteract this phenomenon improving the total weight gain and feed conversion efficiency of free-range birds. In addition, pasture intake decreased carcass yield and meat pH and improved breast skin pigmentation. Moreover, consumption of the leguminous pasture had a marginal influence on the profiles of the vitamin E homologues and on the cholesterol contents of broiler meat. In addition, the consumption of a leguminous-based pasture had a major influence in the fatty acid profile of broiler meat. Although pasture intake did not affect meat contents in LA, levels of n-3 PUFA (ALA, EPA, DPA and DHA) in breast meat were significantly higher in animals consuming the leguminous biomass, which suggest an important deposition of ALA and the conversion of this n-3 precursor to its derivatives in these birds.

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CHAPTER 5 IMPROVING THE LIPID NUTRITIVE VALUE OF POULTRY MEAT THROUGH THE INCORPORATION OF A DEHYDRATED LEGUMINOUS-BASED FORAGE IN THE DIET FOR BROILER CHICKS

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ABSTRACT

Dehydrated forages are assumed to be good sources of ALA and lipid-soluble antioxidant compounds (vitamin E homologues and β-carotene). The effects of including a dehydrated leguminous-based forage in a typical diet for broiler chicken, on performance, meat quality and fatty acid composition were evaluated. One hundred and sixty one-day-old male commercial broiler chicks (Ross 308) were housed in 20 battery brooders. During the 28-d growth period, the animals were fed *ad libitum* with a typical maize-soybean high-energy feed having access or not to a dehydrated leguminous-based forage provided in a separate feeder. The results revealed that dehydrated forage intake (which was 11.1% of the total intake) had no impact in broiler performance (P>0.05). The capacity of ingested forage to modulate broiler meat fatty acid profiles and the meat content in total cholesterol, tocopherols, tocotrienols and β-carotene was investigated in broiler chicks slaughtered at day 28. Dehydrated forage consumption had no effect on the lipid-soluble antioxidant compounds and cholesterol contents of broiler meat but performed a significant effect on meat fatty acid profile. Although forage intake did not affect the LA and ALA contents in poultry meat, the levels of n-3 long-chain polyunsaturated fatty acids [EPA (P=0.004), DPA (P=0.010) and DHA (P=0.007)] in breast meat were significantly higher in animals consuming leguminous biomass, which suggest a higher conversion of ALA into its derivatives in these birds. Overall, the data confirms that incorporation of a dehydrated leguminous-based forage in the diet for broiler chicks results in more favourable P/S and n-6/n-3 nutritional ratios for animals slaughter at earlier stages of grow.

***Key words**:* broiler, leguminous-based forage, meat quality, fatty acid profile

5.1 INTRODUCTION

Low ratios of P/S in Western diets have been considered as major risk factors for cardiovascular diseases, which are among the most important causes of human mortality in developed countries (Hu *et al*., 2001; Ganji *et al*., 2003). In addition, PUFA contents of modern diets are low in n-3 fatty acids leading to high n-6/n-3 fatty acid ratios (Simopoulos, 2002). The imbalance in the n-6 *vs.* n-3 proportion is responsible for the pathogenesis of many diseases, including cardiovascular disease, cancer, and inflammatory and autoimmune diseases (Simopoulos, 2004). In addition, it has been shown that consumption of long-chain (LC; C≥20) n-3 fatty acids, such as EPA and DHA that are vital components in the retina and the membrane phospholipids of the brain, may reduce the risk of coronary heart disease (Rymer & Givens, 2005). Considering the above discussion, it is widely acknowledged that there is an urgent need to return to a balanced fatty acid diet by improving the intake of polyunsaturated fats and n-3 fatty acids (Simopoulos, 2002).

Poultry meat has been considered as one of the main sources of PUFA for human diets, in particular n-3 PUFA (Sioen *et al*., 2006; Howe *et al*., 2006). It has been shown, that the content of poultry meat in n-3 fatty acids, particularly in ALA, can be readily improved by increasing the levels of n-3 PUFA in poultry diets through the incorporation of linseed oil (López-Ferrer *et al*., 1999, 2001a) and/or oily fish by-products (Hulan *et al*., 1988; López-Ferrer *et al*., 2001b). However, a decrease in flavour quality has been reported for these products due to an overall higher meat susceptibility to lipid oxidation (Manilla & Husveth, 1999; Bou *et al*., 2001). It is well known that green pastures are a good source of ALA and pasture consumption leads, in ruminants, to higher contents of this fatty acid in meat while decreasing the n-6/n-3 fatty acid ratio (Wood & Enser, 1997; O'Sullivan *et al*., 2004). In a recent work developed in our laboratory, it was shown that pasture intake promotes the consumption of a cereal-based feed available for *ad libitum* consumption by free-range broilers, leading to an increased body weight (Ponte *et al.*, 2008a; see section 2.1). Pasture intake, which ranged between 2.5 to 4.5% of the total dry matter consumed, had a small impact on the fatty acid profile of broiler breast meat, although the levels of EPA in poultry meat were higher in animals consuming pasture. In addition, lower levels of the n-3 precursor ALA were observed in meat from these animals, suggesting a

higher conversion of ALA into EPA and deposition on birds with access to pasture (Ponte *et al.*, 2008b). However, the effects of including dehydrated leguminous-based forages in typical broiler diets exploited under intensive production systems on poultry performance and meat fatty acid composition remain to be evaluated.

Leguminous-based forages may be considered as a source of fibre and protein for growing broilers. Moreover, forages are also a good source of tocopherols and tocotrienols, the natural diterpenes with vitamin E activity, which is the primary lipid-soluble antioxidant in biological systems (Kerry *et al*., 2000). Tocotrienols are also known to help lower plasma cholesterol levels (Qureshi *et al*., 1997). Antioxidant supplementation of feed is an efficient method for increasing meat oxidative stability (Maraschiello *et al*., 1999), although the various vitamin E forms are known to present different antioxidant potencies (Bourgeois, 1992). In addition, β-carotene, a pro-vitamin A compound abundant in forages, is the predominant carotenoid in meat and meat products (Mortensen *et al.*, 2000). This carotenoid has been suggested to function as a dietary lipid-soluble antioxidant, with an important role in controlling oxidatively induced diseases, such as cancer and atherosclerosis (Decker *et al.*, 2000). The contribution of dehydrated leguminous based forage vitamin E homologues and β-carotene for the oxidative stability of meat from chicken remains, however, to be established. Finally, meat provides from one third to one half of the daily-recommended cholesterol intake (300 mg, WHO), which seems to be directly associated to a greater risk of hypercholesterolemia (Chizzolini et al., 1999). It has been previously shown that the inclusion of dehydrated leguminous forages in broiler diets contributes to decrease cholesterol contents of broiler meat (Ponte *et al.*, 2004). The objective of the research reported here was to establish the impact of providing broiler chicken free access to a dehydrated leguminous-based forage, offered in a separate feeder for *ad libitum* consumption, on bird performance, meat fatty acid profile, and cholesterol, and lipid-soluble antioxidant vitamins (vitamin E homologues and β-carotene) contents, of animals of a fast-growing genotype exploited under an intensive production system.

5.2 MATERIAL AND METHODS

5.2.1 Animals, diets and management

One hundred and sixty one-day-old male chicks Ross 308 were housed in 20 battery brooders (Figure 5.1). The birds were randomly assigned into one of the two treatments with 10 replicates of eight birds per treatment. The two treatments consisted on providing separate (with forage) or not providing access (without forage) to a dehydrated leguminous based forage available in a separate feeder. For both treatments, water and a cereal-based feed were available *ad libitum* throughout the experiment and were provided in two automatic drinking nipples and in an individual feeder, respectively. The composition of the basal diet used on these studies, which was formulated to contain adequate nutrient levels as defined by the NRC (1994), is presented in Table 5.1. Dehydrated forage was obtained from a pasture based in italian ryegrass (*Lolium multiflorum*) and balansa clover (*Trifolium michelianum*), harvested in the flowering state, dehydrated, ground and pelleted (Figure 5.2). The dehydrated forage was presented in 4 mm diameter pellets and provided in separate feeder to the birds from of the "with forage" group. The proximate chemical composition of the dehydrated leguminous based forage is presented in Table 5.2. Weekly, cereal based feed consumption, dehydrated forage intake 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. At the end of the experiment, at day 28, one bird per cage was slaughtered by an intravenous injection of an aqueous solution of 125 mg Tiopental Brown (B. Braun Medical SA, Barcelona, Spain) and the various gastrointestinal compartments were weighed and measured. Skinless breast meat samples (approximately 10 g) were collected for determining total lipids, fatty acid composition, total cholesterol, vitamin E compounds and β-carotene, ground using a food processor (3 × 5 s), vacuum packed and stored at -80 ºC until required.

Figure 5.1 Ross 308 chicken housed in battery brooders in temperature controlled room where experiment took place.

Figure 5.2 Aspect of the leguminous-based forage harvested in the flowering state (Panel A), and the resulting dehydrated and pelleted feed (Panel B).

Table 5.1 Ingredient composition and calculated analysis of the cereal-based feed.

Ingredients	**%**
Corn	59.3
Soybean meal 47%	24.3
Whole soybean	13.3
Salt	0.23
Calcium carbonate	0.80
Dicalcium phosphate	1.13
Choline 75%	0.09
DL-Methionine	0.24
Lysine	0.07
Mineral and vitamin premix [1]	0.54
Calculated nutrient content	
Energy (kcal/kg DM)	3000
Crude protein (%)	21.2
Ca (%)	0.75
P available (%)	0.30
Lysine (%)	1.20
Met+Cys (%)	0.92

[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, 100 mg; Zn, 80 mg; Co, 0.2 mg; I, 1.0 mg; Se, 0.3 mg; monensin, 100 ppm.

Table 5.2 Chemical composition of the dehydrated leguminous-based forage (% DM).

	Dehydrated leguminous-based forage
Dry matter	93.25
Crude protein	20.78
Ether extract	2.66
NDF	46.90
ADF	24.59
ADL	4.49

5.2.2 Determination of total lipids

Meat samples were lyophilized (-60 ºC and 2.0 hPa) to constant weight using an Edwards Modulyo lyophilizer (Edwads High Vaccum International, UK), maintained desiccated at room temperature and analyzed within two weeks. For total lipid determination, intramuscular fat was extracted as described by Alfaia *et al*. (2006) from the lyophilized samples (0.25 g). Total lipids were measured gravimetrically, in duplicate, by weighing the fatty residue obtained after solvent evaporation.

5.2.3 Determination of fatty acid composition

Intramuscular fat of lyophilized samples (0.25 g), cereal-based feed or pasture (0.10 g of dry matter) were dissolved in 1 ml of dry toluene. Then, fatty acids were converted to methyl esters (FAME) by base-catalyzed transesterification with sodium methoxide for 2 h at 30 ºC. The fatty acid composition was determined by gas chromatography of FAME, performed with a gas chromatograph Varian 3800 (Varian Inc, Walnut Creek, CA, USA) equipped with a flame ionization detector and an OmegaWax 250 (Supelco, Bellefont, CA, USA) capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness). The chromatographic conditions were as follows: injector temperature, 250 ºC; detector temperature, 280 ºC; helium was used as carrier gas and the split ratio was 1:20. The gas chromatograph oven temperature was programmed to start at 150 ºC (maintained for 15 min) followed by a 3 ºC/min ramp to 220 ºC (maintained for 20 min). Identification was accomplished by comparing the retention times of peaks from samples with those of FAME standard mixtures. 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 %, using the corrected response factor of each fatty acid (ES ISO 5508, 1990). Fatty acids were expressed as gravimetric contents (mg g^{-1} feed) or as a percentage of the sum of identified fatty acids (% w/w).

5.2.4 Quantification of total cholesterol, β-carotene, tocopherols and tocotrienols

The simultaneous determination of total cholesterol, β-carotene, tocopherols and tocotrienols was performed as described by Prates *et al*. (2006). The method involves a direct saponification of the fresh meat (0.75 g), high-energy feed or pasture (0.10 g of dry matter), a single *n*-hexane extraction and the analysis of the extracted compounds by normal-phase HPLC, using fluorescence (tocopherols and tocotrienols) and UV-Vis photodiode array (cholesterol and β-carotene) detections in tandem. The contents of total cholesterol, β-carotene, 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.

5.2.5 Analytical procedures

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).

5.2.6 Statistical analysis

Statistical analysis was conducted by analysis of variance using SAS with the GLM procedure (SAS Institute, 2004). The experimental unit considered was the pen. Unless otherwise stated, differences were considered significant when $P<0.05$.

5.3 RESULTS AND DISCUSSION

The effects that derive from the incorporation of dehydrated leguminous-based forages in broiler diets on bird performance, meat cholesterol, lipid-soluble antioxidant vitamins and

fatty acid profile of animals of a fast-growing genotype exploited under an intensive production system remain largely unknown. Here an experiment was conducted to evaluate the importance of forage consumption, offered free choice in a separate feeder, in the performance, meat fat and vitamin E composition of broilers. In order to effectively access the importance of forage intake, control birds were exposed to the same experimental conditions, without access to the dehydrated leguminous-based forage. Therefore, nutrient supply in the control birds derived exclusively from the cereal-based feed, which was available, for both groups, *ad libitum*.

5.3.1 Dehydrated leguminous-based forage and cereal-based feed composition

Dehydrated forage displayed relatively high crude protein content as a consequence of the predominance of leguminous species (Table 5.2). As expected, NDF was the main organic component of the forage that presented a residual percentage of ADL. In addition, the fatty acid composition of the cereal-based feed and of the dehydrated leguminous-based forage is presented in Table 5.3. Total fatty acids were higher in the cereal-based feed than in dehydrated forage. As expected, LA was the major fatty acid in the cereal-based diet, while ALA predominates in the leguminous-based forage. Palmitic acid (16:0) is relatively abundant in both feeds, although with higher levels in the forage relative to cereal-based feed. In contrast, the cereal-based feed contained higher percentages of oleic acid (18:1n-9) when compared with the forage. In addition, EPA and DHA were residual in all the feeds analyzed (data not shown). Finally, the forage presented a LA/ALA ratio of 0.58, while the cereal-based feed depicted a LA/ALA ratio of 10.3.

Table 5.3 Total fatty acids (mg g^{-1} dry matter), fatty acid composition (% w/w), diterpenes (tocopherols and tocotrienols) and β-carotene (μg g^{-1} dry matter) of the cereal-based feed and of the dehydrated leguminous-based forage.

	Cereal-based feed[1]	**Dehydrated leguminous-based forage**
Total fatty acids	47.69	6.89
Fatty acids		
14:0	0.15	1.29
16:0	12.71	22.09
16:1n-7	0.18	0.26
17:0	0.14	0.72
18:0	3.47	2.22
18:1n-9	22.13	9.66
18:2n-6	55.44	22.68
18:3n-3	5.39	39.28
20:0	0.39	1.80
Diterpenes		
α-Tocopherol	56.744	53.464
α-Tocotrienol	22.425	15.632
β-Tocopherol	1.143	1.636
γ-Tocopherol[2]	55.284	7.581
γ-Tocotrienol	43.885	40.556
δ-Tocopherol	10.748	1.129
δ-Tocotrienol	4.928	4.414
β-Carotene	0.595	4.559

[1] The cereal-based feed was supplemented with α-tocopherol (20 mg kg^{-1}); [2] Co-eluted with small amounts of β-tocotrienol.

The content of feedstuffs in diterpenes (tocopherols and tocotrienols) is also presented in Table 5.3. Although γ-tocopherol was co-eluted with a minor proportion of β-tocotrienol, a complete profile of vitamin E compounds was obtained. The α- and γ-tocopherols are the most abundant vitamin E homologues in the cereal-based diet, in accordance with the exogenous supplementation of α-tocopherol to the cereal-based feed, whereas α-tocopherol and γ-tocotrienol predominate in the leguminous-based forage. It is well known that tocotrienols have different antioxidant potencies and biological activities when

compared with tocopherols and, therefore, the determination of all vitamin E molecules in feed is critical. In addition, the forage presented significant levels of β-carotene, when compared with the cereal-based feed, which displayed a lower level of this lipid-soluble antioxidant pro-vitamin.

5.3.2 Bird performance

The mortality rate during the experiment was low (2.5%) and was not related with the treatments. The results of bird performance and feed intake, expressed as body weight, weight gain, intake of cereal based feed and dehydrated forage and feed conversion ratios are summarized in Table 5.4 and Table 5.5. The proportion of dehydrated forage consumed on total intake is also presented in Table 5.4. The provision of the dehydrated leguminous-based forage free choice to the broiler chicken had no significant effects on the birds' final body weights. Although weight gains of birds consuming dehydrated forage were significantly lower than birds without access to forage in the first and second week of the experiment, no differences were observed in weight gains in the last two weeks and in the total weight gain. In addition, in the early stage of the experiment, the access to forage had a negative impact on the cereal-based feed intake. However, differences in feed intake from 14 to 21 d and in total feed intake between the two experimental groups were not significant. There were no differences between the feed conversion ratios of animals subjected to the two treatments (computed considering only with cereal-based feed intake), suggesting that bird performance primarily depends on the intake of the cereal-based feed (Table 5.5). However, when the intake of the dehydrated forage was considered, FCR are significantly higher in birds with access to the fibrous biomass (Table 5.5). Although the proportion of forage in total intake varied during the experiment, it was found that dehydrated leguminous based forage represented 11.1 %, on a DM basis, of the total feed intake in the animals with *ad libitum* access to the forage. The higher proportion of dehydrated forage intake observed in the third week of the experiment had no negative impact on the consumption of the cereal based feed and, consequently, did not decrease weight gains.

Table 5.4 Performance broilers fed on a cereal-based feed supplemented or not supplemented with a dehydrated leguminous-based forage.

	With forage	Without forage	SEM	Significance
Body weight (g)				
0 d	42.7	42.2	0.194	ns
7 d	129.5	134.9	1.98	ns
14 d	314.3	334.0	4.94	*
21 d	587.9	621.9	11.60	*
28 d	1025.9	1055.0	20.79	ns
Weight gain (g)				
0-7d	87.0	93.0	1.82	*
7-14d	184.5	198.9	3.46	**
14-21d	273.6	287.9	7.51	ns
21-28d	435.2	433.1	10.71	ns
0-28d	983.3	1012.8	20.76	ns
Intake (g)				
Cereal based feed				
0-7d	119.1	131.3	2.96	**
7-14d	309.1	342.1	5.56	***
14-21d	502.3	517.5	11.31	ns
21-28d	831.0	831.4	16.61	ns
0-28d	1761.3	1821.8	27.85	ns
Dehydrated forage				
0-7d	11.7	-	1.34	-
7-14d	35.6	-	2.72	-
14-21d	98.0	-	3.65	-
21-28d	75.3	-	9.92	-
0-28d	219.9	-	14.06	-
DF/TI [1](%)				
0-7d	8.55	-	1.084	-
7-14d	10.29	-	0.738	-
14-21d	16.35	-	0.650	-
21-28d	8.23	-	1.021	-
0-28d	11.09	-	0.668	-

Significance: ns, P>0.05; *, P<0.05; ** P<0.01 *** P<0.001; Means with the same row bearing different superscripts are significantly different; SEM, standard error of mean.
[1]DF/TI = dehydrated forage intake/total intake (CBF+DF).

Table 5.5 Feed conversion ratio of broilers fed on a cereal-based feed, which were provided separate (with forage) or not provided (without forage) access to a dehydrated leguminous-based forage. FCR for birds with access to the dehydrate forage was calculated considering the intake of the cereal based feed (CBF) solely or in conjunction with the forage.

	With forage		Without forage	SEM	Significance
	TI[2]	CBF			
FCR[1]					
0-7d	1.50[a]	1.37[b]	1.41[b]	0.023	**
7-14d	1.87[a]	1.68[b]	1.72[b]	0.028	***
14-21d	2.20[a]	1.84[b]	1.80[b]	0.034	***
21-28d	2.09[a]	1.91[b]	1.93[b]	0.044	**
0-28d	2.02[a]	1.79[b]	1.80[b]	0.024	***

Significance: ns, P>0.05; *, P<0.05; ** P<0.01 *** P<0.001; Means with the same row bearing different superscripts are significantly different; SEM, standard error of mean. [1]FCR = cereal based feed intake per pen / total weight gain per pen. [2]TI (total intake) = CBF+DF

The relative size and length of the different sections of the gastrointestinal tract of birds of the two treatments were evaluated and are displayed in Table 5.6. The data suggested that these variables were not affected by the inclusion of the dehydrated leguminous based forage in the diet. Similar observations were made in previous studies in animals fed on diets displaying high levels of insoluble fibre (Preston *et al.* 2000; Wu & Ravindran, 2004; Mourão *et al.*, 2008). In contrast, the intake of soluble fibres is known to cause a significant enlargement in the gastrointestinal tract of birds, as a result of an increased development of the small intestine (Mourão, 2000; Brenes *et al.*, 1993). Taken together, the results suggest that incorporation of a fibrous-rich dehydrated forage in the diets for broiler chicken, from days 1-28, had no significant impact on bird performance in the end of the experiment.

Table 5.6 Relative weight and length of GI tract of free-range broilers fed on a cereal-based feed supplemented or not supplemented with dehydrated leguminous-based forage.

	With forage	Without forage	SEM	Significance
Relative weight (g/100g BW)				
Crop	0.333	0.378	0.0213	ns
Gizzard	2.403	2.364	0.1305	ns
Liver	2.677	2.638	0.0677	ns
Relative length (cm/100g BW)				
Duodenum	2.272	2.213	0.0730	ns
Jejunum+ileum	12.270	11.941	0.3634	ns
Caecum	1.580	1.481	0.0557	ns

Significance: ns, $P>0.05$; SEM, standard error of mean.

5.3.3 Cholesterol and fatty acid composition of meat

Consumption of the dehydrated leguminous forage had no impact ($P>0.05$) on the meat total lipids and total cholesterol content (Table 5.7). However, both chicken meats are lean, based on the Food Advisory Committee (1990) criteria (<5% fat), and depict median contents of total cholesterol (0.58-0.59 mg/g), when compared with those reviewed by Chizzolini *et al.* (1999) for beef.

Data concerning the fatty acid composition of breast meat are also presented in Table 5.7. The predominant fatty acids in chicken meats of both treatments were palmitic and stearic (18:0) acids as SFA, oleic acid as MUFA, and LA and arachidonic acid (20:4n-6) as PUFA. Oleic and palmitic acids were the most abundant fatty acids in meats under analysis. Dehydrated forage consumption induced significant effects on the fatty acid profile of broiler meats. Although forage intake had no effect on total SFA, a slight but significant increase was observed in stearic acid (18:0) as well as significant decreases were observed in the percentages of palmitoleic (16:1n-7) and oleic (18:1n-9) acids. These changes strongly suggest that the activity of stearoyl-CoA desaturase (SCD) is depressed in animals of the forage consuming group. It is well known that the SCD expression may be regulated by nutritional factors (Rosebrough *et al.*, 2005). Considering that

cereal-based feed intake did not change between groups, the likely depression of SCD should be somehow directly associated with forage intake. Moreover, consumption of dehydrated forage did not affect the percentages of LA and ALA in broiler meat (P>0.05). In fact, ALA present in pasture is in the esterified form in structural lipids, including galactolipids from chloroplasts (Gurr, 1984). Thus, the broiler digestive system may not be able to promote exposure of the structural lipids and may lack galactolipase activity to free the ALA from galactolipids in a significant extent. In contrast, consumption of dehydrated forage increased the proportion of most of the C20 and C22 PUFA and hence total PUFA (P=0.020) and the P/S ratio (P=0.036). Specifically, dehydrated forage intake influenced the levels of arachidonic acid (20:4n-6), 20:3n-6 and 22:4n-6 in broiler meat, though no significant increase was observed in total n-6 fatty acids.

Consumption of the dehydrated leguminous based forage, displaying a low LA/ALA ratio (0.58), contributed to slightly enhance the n-3 PUFA (P=0.001) content and decrease (P=0.001) the n-6/n-3 ratio in broiler meat (from 21.62 to 18.53). However, the reported decrease in the n-6/n-3 ratio has very little value regarding the current nutritional recommendations for human diets, which suggest that this ratio should not exceed 4.0 (British Department of Health, 1994). However, the levels of the LC n-3 PUFA, EPA (P=0.004), docosapentaenoic acid (DPA; 22:5n-3; P=0.010) and DHA (P=0.007), were higher in birds consuming forage. Therefore, since no differences were obtained in ALA content as a consequence of forage intake, the data suggests a larger conversion, by desaturation and elongation, of ALA to its derivatives in broilers with access to dehydrated forage. Similar results have been obtained in previous studies using linseed oil (López-Ferrer *et al.*, 2001a) and leguminous biomass (Ponte *et al.*, 2008b; Mourão *et al.*, 2008). This finding supports the ability of the chickens to convert ALA to its derivatives when it is present in the diet. However the levels of EPA, DPA and DHA in the forage consuming broiler meat are much lower when compared with the percentages of the LC n-3 fatty acids reported in meat originated in birds supplemented with 2-4% of fish oil (López-Ferrer *et al.*, 2001a). Nevertheless, data from a previous study suggests that dehydrated leguminous-based forage intake did not create off-flavours in poultry meat (Ponte *et al.*, 2004), such as has been reported for meat from broilers fed on diets containing fish products (López-Ferrer *et al.*, 1999).

Table 5.7 Total lipids (mg g^{-1}), cholesterol (mg g^{-1}), fatty acid composition (%w/w) partial sums of fatty acids (%w/w) and nutritional values of meat from broilers fed on a cereal-based feed, which were provided separate (with forage) or not provided (without forage) access to a dehydrated leguminous-based forage.

	With forage	Without forage	SEM	Significance
Total lipids	3.64	3.96	0.183	ns
Cholesterol	0.584	0.586	0.0112	ns
Fatty acids				
14:0	0.33	0.35	0.009	ns
15:0	0.15	0.14	0.029	ns
16:0	22.04	22.21	0.141	ns
16:1n-7	2.68	3.24	0.153	*
17:0	0.18	0.17	0.017	ns
18:0	9.74	9.00	0.187	**
18:1n-9	30.12	32.42	0.688	*
18:2n-6	20.97	20.82	0.218	ns
20:0	0.09	0.11	0.008	ns
18:3n-6	0.21	0.21	0.014	ns
20:1n-9	0.51	0.53	0.023	ns
18:3n-3	0.52	0.50	0.014	ns
20:2n-6	1.03	0.93	0.044	ns
20:3n-6	1.33	1.16	0.053	*
20:4n-6	6.55	5.38	0.314	*
20:3n-3	0.05	0.04	0.004	ns
20:5n-3	0.19	0.14	0.001	**
22:2n-6	0.15	0.14	0.006	ns
22:4n-6	2.11	1.70	0.113	*
22:5n-3	0.54	0.42	0.031	**
22:6n-3	0.43	0.31	0.027	**
Partial sums				
SFA	32.54	31.98	0.232	ns
MUFA	15.66	17.60	4.686	ns
PUFA	34.07	31.75	0.654	*
n-6	31.32	29.72	0.646	ns
n-3	1.700	1.390	0.059	***
Ratios				
P/S	1.05	0.99	0.017	**
n-6/n-3	18.53	21.62	0.566	***

Significance: ns, P>0.05; *, P<0.05; ** P<0.01 *** P<0.001; Means with the same row bearing different superscripts are significantly different; SEM, standard error of mean.

Overall, the data suggests that consumption of a dehydrated forage, offered *ad libitum* and in a separate feeder to broiler chicken, did not contribute to improve the levels of ALA in breast meat, while desaturation and elongation of this fatty acid precursor contributes to the synthesis of its LC family derivatives. Therefore, these results indicate that free access to dehydrated leguminous-based forage for fast-growing broilers with a cereal-based feed available *ad libitum*, is able to improve the contents of LC n-3 PUFA of broiler meat, although still not to a level that may be nutritionally valuable. Under these circumstances, a combined supplementation of leguminous based forage and other LC-PUFA rich feedstuff may be an alternative route to improve meat nutritive value with less unfavourable effects on meat sensory quality.

5.3.4 Tocopherols, tocotrienols and β-carotene of meat

The diterpenes α-tocopherol and γ-tocotrienol were the major vitamin E homologues detected in breast meats (Table 5.8). In addition, small contents of γ-tocopherol, which co-eluted with a minor proportion of β-tocotrienol and β-tocopherol were also identified. The prevalence of α-tocopherol in meat is well known and is due to the more than tenfold preference of the tocopherol-binding protein for α-tocopherol, relative to γ-homologues, which are the most common vitamin E molecules in plant foods (Decker *et al.*, 2000). All the vitamin E homologues scored higher values in meat from birds with access to dehydrated forage. However, no significant improvement was obtained. In addition, although the leguminous based forage presented significant levels of β-carotene (4.56 μg g^{-1} dry matter), no effect was observed in the content of this lipid-soluble antioxidant pro-vitamin in the meat samples from birds consuming forage.

Table 5.8 Diterpenes (tocopherols and tocotrienols) and β-carotene contents (μg g^{-1} meat) of meat from broilers with and without access to dehydrated leguminous-based forage.

	With Forage	Without Forage	SEM	Significance
Diterpenes				
α-Tocopherol	8.159	7.913	0.3107	ns
α-Tocotrienol	1.895	1.294	0.2679	ns
β-Tocopherol	0.089	0.059	0.0129	ns
γ-Tocopherol[1]	1.612	1.604	0.1100	ns
γ-Tocotrienol	4.923	3.233	0.8371	ns
δ-Tocopherol	0.677	0.367	0.1316	ns
β-Carotene	0.043	0.037	0.0064	ns

Significance: ns, P>0.05; SEM, standard error of mean. 1 Co-eluted with small amounts of β-tocotrienol.

In conclusion, in this study the consumption of a dehydrated leguminous-based forage, offered free choice and *ad libitum*, by broilers of a fast-growing genotype exploited under an intensive production system had no effect on broiler performance. In addition, the dietary dehydrated forage had no influence on the profiles of vitamin E homologues and on the cholesterol content of broiler meat. However, the intake of the dehydrated forage had a major influence in the fatty acid profile of broiler meat. Although the dietary forage had no effect on the poultry meat contents of LA and ALA, the levels of LC n-3 PUFA (EPA, DPA and DHA) in breast meat were significantly higher in animals consuming the leguminous biomass. These data suggest a higher conversion of ALA into its derivatives in these birds, which contributes to more favourable meat nutritional fatty acid ratios. Together the data suggest that dehydrated leguminous-based forages can be used to improve the nutritional quality of meat from broilers slaughter at earlier stages of grow without affecting poultry performance.

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CHAPTER 6 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

In recent years, consumer concern on health, environmental protection and sustainable agriculture have leaded to an increasing interest in specialty poultry products, such as those originated from free-range or organic production systems. Pasture consumption is one of the main attributes of alternative production systems, although its importance for broiler performance and poultry meat quality remain largely unknown. Additionally, the impact of diet supplementation with fibre degrading enzymes on broiler performance in free-range production systems remains to be established. The study reported here aimed to elucidate several unsolved aspects concerning the production of free-range poultry in pastures, and to evaluate its impact on bird performance and meat quality.

The effect of pasture intake and enzyme supplementation on performance, meat quality and sensory attributes of pastured poultry was evaluated. The data suggest that pasture intake promotes growth by improving the consumption of the cereal-based feed, although the levels of forage intake were low. In contrast, the supplementation of a cereal-based diet for pastured broilers with high levels of microbial cellulases and hemicellulases did not improve broiler performance. The data concerning meat sensory attributes suggest that pasture intake, even when consumed at reduced levels, generates broiler meat with higher degrees of consumer acceptability. Moreover, data revealed that the slow growing genotype produces meat with higher sensory attributes, when compared with the fast growing genotype, while pasture intake can further improve its intrinsic overall acceptance. Together, the data suggest that according to the consumers, poultry products originated from alternative pastured-based systems present higher standards of sensory quality.

The impact of pasture consumption on the fatty acid composition, cholesterol, tocopherol and tocotrienol contents of meat from pastured broiler chicken was evaluated. Low levels of pasture intake had a low impact on cholesterol content, fatty acid and vitamin E homologue profiles of meat from free-range broilers, suggesting that meat properties were more dependent on the composition of the cereal-based feed available for *ad libitum* consumption. However, animals with access to pasture presented lower levels of LA and ALA in breast meat. Although pastures presented high contents of ALA, decreased levels of this precursor of long-chain n-3 fatty acids were observed in breast meat form birds foraging on leguminous-based pastures. Additionally, in spring, the levels of EPA in

breast meat were significantly higher in animals consuming pastures, which suggest a higher conversion of ALA into EPA in these birds. Meat lipid quality generated in commercial intensive production and free-range systems was also investigated. When compared with meat from slow growing genotypes obtained in low intensive production systems with slaughtering at day 81, meat from birds raised intensively and slaughtered at day 35 presented higher levels of PUFA and n-3 fatty acids and lower levels of SFA, which leaded to improved nutritional indices.

The capacity of a bifunctional recombinant derivative of *Ct*Lic26A-Cel5E from *Clostridium thermocellum*, to enhance the nutritive value of a barley based diet for free-range pastured birds of a slow-growing genotype was investigated. The individual recombinant bi-modular cellulase from *C. thermocellum* was unable to improve the nutritive value of a barley-based feed available *ad libitum* for pastured broilers of a slow-growing genotype. The data reported here suggest that, although the enzyme suffers proteolysis on the GI tract, it retains its full catalytic activity, suggesting that a lack of response to enzyme supplementation does not relate to enzyme degradation or inhibition. However, non-supplemented animals presented significant levels of β-glucanases activity in the GI tract that have both a plant and a microbial origin. Microbial flora is, however, only well established at later stages of the animals growth. Together, the data suggest that the moderate levels of cellulase activity observed in the crop of non-supplemented animals is of plant and microbial origin and are sufficient to degrade, partial or totally, the anti-nutritive β-glucans present in barley based diets. Nevertheless, further work is required to establish if functional endogenous β-glucanases are expressed by most barley varieties or are restricted to specific lots of this cereal, which would have both practical and economical importance in animal feed production.

The effects of restricting the intake of a cereal-based feed on the consumption of a leguminous-based pasture and, consequently, on poultry performance, meat quality and fatty acid, cholesterol, and lipid-soluble antioxidant vitamins (vitamin E homologues and β-carotene) content of the meat were evaluated. In the study reported here, the imposed restriction on the intake of a cereal-based feed for free-range broilers leaded to a higher proportion of pasture intake in the overall total intake, although the absolute pasture consumption was equivalent in all groups. Feed restriction had a negative effect on broiler

performance and growth rate achieved by the broilers was below the levels expected for the genotype. However, pasture consumption had the capacity to contribute to attenuate this detrimental effect, improving the total weight gain and feed conversion efficiency of pastured birds. Additionally, pasture intake decreased carcass yield and meat pH and improved breast skin pigmentation, indicating a higher deposition of natural pigments present in the leguminous-based pasture. In addition, the consumption of a leguminous-based pasture had a major influence in the FA composition of broiler meat, leading to significant increases in most PUFA. Although pasture intake did not affect meat contents in LA, the levels of n-3 PUFA (ALA, EPA, DPA and DHA) in breast meat were significantly higher in animals consuming the leguminous biomass. Since LC n-3 PUFA were present in trace levels in both the cereal-based feed and in pasture, the data presented here suggest a significant conversion of ALA to its long-chain derivatives in broilers with access to pasture, by desaturation and elongation. Moreover, consumption of the leguminous pasture had a minor influence on the profiles of the vitamin E homologues and on the cholesterol contents of broiler meat.

Finally, the impact of including a dehydrated leguminous-based forage in broiler diets exploited under the conventional intensive system on bird performance and on meat fatty acid, and cholesterol, and lipid-soluble antioxidant vitamins (vitamin E homologues and β-carotene) contents of animals of a fast-growing genotype was evaluated. The consumption of a dehydrated leguminous-based forage, offered free choice and *ad libitum*, by broilers of a fast-growing genotype exploited under an intensive production system had no effect on broiler performance. In addition, the dietary dehydrated forage had no influence on the profile of vitamin E homologues and on the cholesterol content of broiler meat. However, the intake of the dehydrated forage had a major influence on the FA composition of broiler meat, leading to significant increases in most PUFA. Although the dietary forage had no effect on the poultry meat contents of LA and ALA, the levels of LC n-3 PUFA (EPA, DPA and DHA) in breast meat were significantly higher in animals consuming the leguminous biomass. These data suggest a higher conversion of ALA into its long-chain derivatives in these birds, which contributed to more favourable meat nutritional fatty acid ratios. Together, the data suggest that dehydrated leguminous-based forages can contribute to improve the nutritional quality of meat from broilers slaughter at

earlier stages of grow without affecting poultry performance.

Globally, the data suggest that although important amounts of ALA are available in both fresh and dehydrated leguminous forages, the deposition of this fatty acids in meat from pastured birds was often not nutritional valuable, although significantly higher than in meat from birds without access to leguminous biomass. Since ALA present in pastures is mostly in the esterified form (in structural lipids), it is possible that the broiler digestive system may not be able to digest structural lipids or may lack the required galactolipase activity to free ALA from galactolipids. Therefore, further work is required to explore the physiological mechanisms allowing and/or restricting the higher depositions of ALA and its long-chain derivatives in meat from chickens with access to forage.

Taken together, the data suggests that low levels of pasture intake contribute to improve bird performance and meat quality. Nonetheless, further work is required to develop methods to improve pasture intake by poultry, to study poultry foraging behaviour, preferred plant species and varieties and alternative production procedures. Moreover, additional efforts are necessary to quantify more accurately the consumption of pasture biomass by chickens, ideally through the implementation of N-alkane methodologies.

The improvements in n-3 PUFA and LC n-3 PUFA contents in poultry meat, as a result of fresh or dehydrated pasture consumption, were significant although relatively minor to be considered as nutritional valuable. Therefore, further work is necessary to explore the possibility of combining supplementation of leguminous-based forage and other LC-PUFA rich feedstuff to improve meat nutritive value with less unfavourable effects on meat sensory quality. Finally, pastures present high levels of antioxidant vitamins, which are responsible for a protective action against meat lipid oxidation. Therefore, additional work is required in order to establish the effect of pasture intake on the meat susceptibility to oxidation.

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