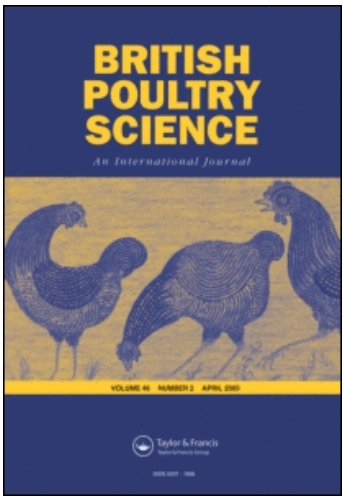

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Crop β-glucanase activity limits the effectiveness of a recombinant cellulase used to supplement a barley-based feed for free-range broilers

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Abstract 1. The supplementation of diets rich in soluble polysaccharides with microbial cellulases and hemicellulases decreases digesta viscosity and promotes broiler performance.

2. In contrast, recent experiments suggest that polysaccharidases are ineffective for improving the nutritive value of pasture biomass used by free-range broilers. However, the feasibility of using cellulases and hemicellulases to improve the utilisation of cereal-based feeds by pastured poultry remains to be established.

3. 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.

4. The results show that supplementation of a barley-based diet with a recombinant β-glucanase had no effect on the performance of free-range broilers, foraging in legume-based diets from d 28 to 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 gastrointestinal tract.

5. Significantly, β-glucanase activity was identified in the crop of non-supplemented animals. The data suggest that endogenous cellulases originated both from the barley-based feed and from the crop microflora.

6. The results presented here suggest that in older birds of slow-growing genotypes associated with free-range production systems, previously unknown sources of β-glucanases, such as the feed and microbial symbiotic microflora, can affect the effectiveness of exogenous enzymes added to the feed.

INTRODUCTION

In general, inclusion of exogenous cellulases and hemicellulases in wheat-, barley- and rye-based diets for monogastric animals improves the efficiency of feed utilisation, enhances growth and contributes to 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 non-starch polysaccharides (NSPs) by a variety of mechanisms. Therefore, by contributing to decreasing the digesta viscosity associated with soluble NSPs, exogenous polysaccharidases have a positive effect on the rate of diffusion of substrates, digestive enzymes and nutrients (White *et al.*, 1981; Fengler and Marquardt, 1988; Bedford *et al.*, 1991; Bedford and Classen, 1992). In addition, cellulases and xylanases may promote the proliferation of beneficial microflora in the final compartments of the gastrointestinal (GI) tract, by increasing the quantity and/or the quality of the substrates available for fermentation (Bedford and Morgan 1996; Apajalahti and Bedford, 1999). Finally, plant cell wall hydrolases may mediate their

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effects by releasing nutrients trapped by the endosperm cell wall that are otherwise unavailable for digestion (Hesselman and Aman, 1986). The action of one or a combination of the above effects may depend on the type of animal, diet and exogenous enzyme used.

Consumer interest in specialty poultry products derived from free-range or organic production systems has been steadily increasing (Fanatico *et al*., 2006). Under these systems, animals have access to the outdoors to promote foraging, feed selection and activity, thus improving birds' general welfare. In Europe, poultry used under these systems may be 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 appropriate for a specialty or gourmet market (Gordon and 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*., 2008). 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 greater incorporation of cereals rich in soluble NSP in the diets of 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 slaughtered at later stages of growth. In addition, it is well established that individual recombinant enzymes are as efficient as complex enzyme mixtures in decreasing 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 bi-functional recombinant derivative of *Ct*Lic26A-Cel5E from *Clostridium thermocellum*, consisting of two catalytic modules expressed as an individual entity, to enhance the nutritive value of a barley-based diet for free-range pastured birds of a slow-growing genotype, from d 28 to 56. The data presented here suggest that the recombinant enzyme is unable to affect the performance of pastured broilers supplemented with a barley-based diet. The enzyme appears as a truncated derivative in the bird's GI tract, due to proteolysis by endogenous digestive enzymes, although retaining full enzyme activity. It is suggested that endogenous crop β-glucanase activity present in birds of slow-growing genotypes at later stages of growth, limits the effectiveness of the exogenous enzymes used to supplement the cereal-based feed.

MATERIALS AND METHODS

Enzyme preparation

*Clostridium 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 1(a). 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 4 Clostridial genes under the control of T7 promoters in the prokaryotic expression vector pET21a (Novagen, Darmstadt, Germany), were used to transform BL21 *E. 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°C 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). *Escherichia coli* cell-free extracts containing the recombinant cellulase A (CelA) from *Ruminococcus albus* were prepared as described by Fontes *et al*. (1995).

Animals, diets and management

The effect of a recombinant β-glucanase on the nutritive value of a barley-based feed for free-range pastured broilers raised between d 28 and 56 was evaluated in an experiment performed in the spring of 2004 at Herdade dos Esquerdos (039° 07·18′ North, 007° 29·36′ West, 318 m above sea level), Vaiamonte, Portugal. During the experiment, the average

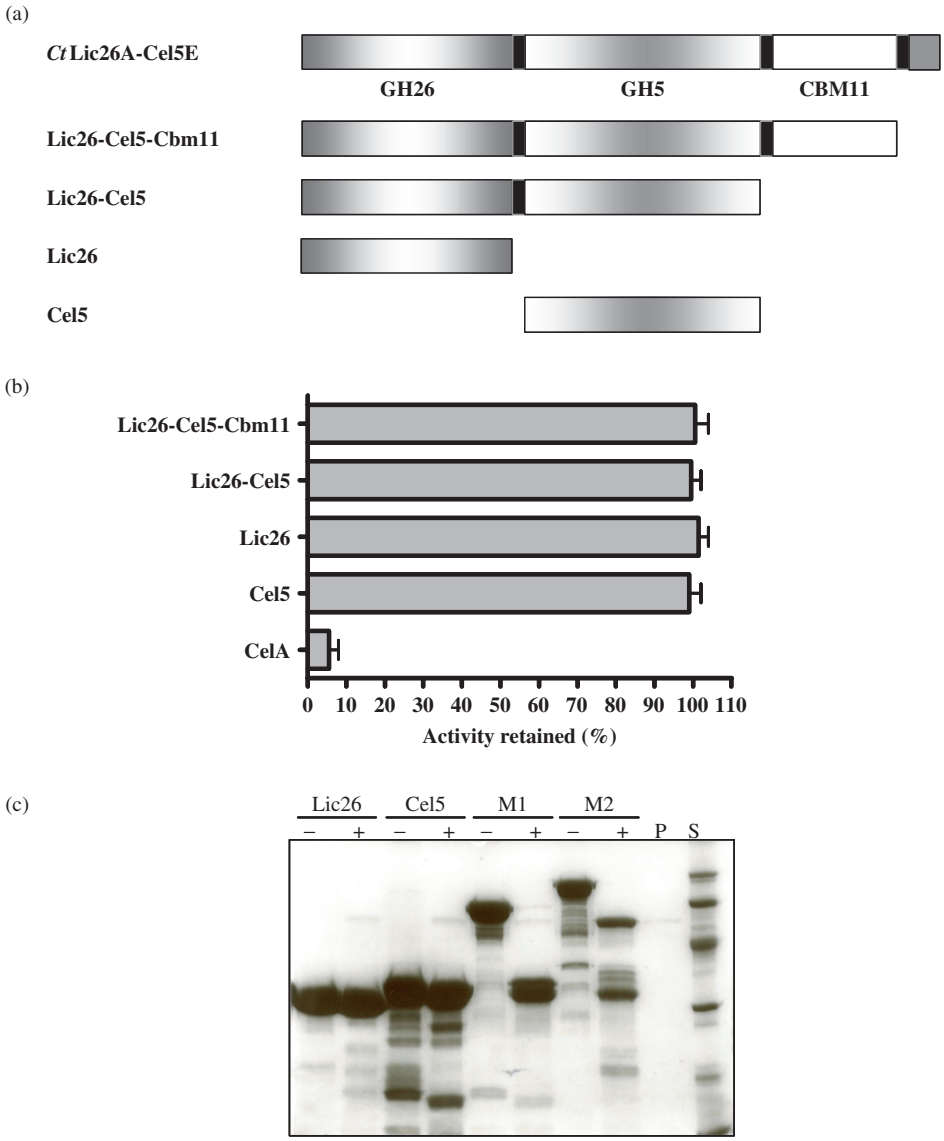

Figure 1. *Molecular architecture of* Ct*Lic26A-Cel5E (Panel A) and resistance of its truncated derivatives to proteolysis (Panels B and C).* Ct*Lic26A-Cel5E is a modular cellulase composed of an N-terminal family 26 glycoside hydrolase catalytic domain (GH26), an additional family 5 glycoside hydrolase catalytic domain (GH5), a family 11 carbohydrate-binding module (CBM11) and a C-terminal dockerin (Panel A). The 4 truncated derivatives of* Ct*Lic26A-Cel5E were incubated with pancreatic proteases. Retained activity (Panel B) and molecular integrity (Panel C) were evaluated after a 3-h incubation period. In Panel B CelA refers to the cellulase A from* Ruminococcus albus *that is susceptible to proteolysis. In Panel C, 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 C refer to the pancreatic proteases and the low molecular mass protein markers, respectively.*

daily mean temperature was 13·2°C (mean of highest temperatures 19·0°C and of the minimum 7·4°C) with 4 d of rain and a total precipitation of 34 mm. A total of 160 28-d-old males (RedBro Cou Nu × RedBro M), vaccinated against Marek disease, were allocated to 16 floorless portable metal outdoor pens (10 birds per pen/replicate), equalising both the mean and the variance of body weight (BW). Until the experiment, the birds were maintained in a conventional indoor facility following standard brooding procedures and fed on a typical maize–soybean meal diet. At d 28, animals were transported to the experimental field and raised for a further 28 d outdoors, confined to the experimental pens that were kept in the pasture until slaughter 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 contact the legume-based pastures directly, promoting forage intake. Approximately one-third of the top of each cage area was covered with transparent white-washed plastic to provide protection against climatic extremes. Water and a barley-based feed were available *ad libitum* throughout the experiments and were provided in two automatic drinking

Table 1. *Ingredient composition and calculated analysis of the cereal-based feed*

Ingredients	g/kg
Barley	619·0
Soybean meal 42%	281·3
Soybean oil	62·8
Salt	2·8
Calcium carbonate	11·5
Dicalcium phosphate[1]	17·2
Choline 60%	0·8
DL-Methionine	1·7
Mineral and vitamin premix[2]	2·0
Calculated nutrient content	
Energy (MJ ME/kg DM)	12·12
Crude protein (g/kg)	184·0
Ether extract (g/kg)	77·0
Crude cellulose (g/kg)	50·0

[1]Contained 200 g/kg Ca and 180 g/kg P.
[2]Mineral–vitamin premix provided the following per kg of diet: biotin 0·5 mg, calcium pantothenate 10 mg, cholecalciferol 0·05 mg, cyanocobalamin 0·12 mg, folic acid 0·5 mg, menadione 2 mg, nicotinic acid 30 mg, pyridoxine 1·7 mg, retinol 2·7 mg, thiamine 1 mg, α-tocopherol 20 mg, riboflavin 4·2 mg, Co 0·2 mg, Cu 10 mg, Fe 80 mg, I 1 mg, Mn 100 mg, Se 0·3 mg, Zn 80 mg, monensin 0·1 g.

nipples and in an individual hanging tube feeder, respectively. The composition of the basal diet used in this study (Table 1), which was provided in pelleted form, was formulated to contain adequate nutrient levels as defined by the NRC (1994).

The birds were randomly assigned into one of the 4 treatments with 4 replicates of 10 birds per treatment. The 4 treatments consisted of 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 rain-fed subterranean clover (*Trifolium subterraneum*) pasture (*Ts*P), in a completely randomised design. Activity of the recombinant enzyme was determined as described below. At d 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 height of the vegetation below 12 to 15 cm. To avoid ingestion of sheep faecal material by birds, sheep faeces were removed from the pasture before the movement of the pens. In order to avoid climate variations, the two pastures used in this experiment, which were installed in the autumn of 2002, were contiguous. The white clover pasture was irrigated during the dry summer season (June to September).

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 d 56, two birds per pen were killed 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 chickens have been reported to feed on a wide range of macro-invertebrates living in the surface soil (Clark and 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 d 56, 6 birds per pen were slaughtered at a commercial processing plant. The carcases were refrigerated for 24 h and weighed. Meat pH was measured as described by Sierra (1973).

Skin colour

The colour of breast skin was evaluated using a Minolta chromameter CR-300. The readings were taken on equivalent positions of the carcases. The tip of the chromameter-measuring head was placed flat against the surface of the skin. For each reading three 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, carcases were frozen at −20°C until analysis.

Analytical procedures

Analyses of dry matter (DM), ether extract, crude protein and dietary fibre were performed according to the methods of the Association of Official Analytical Chemists (1980). Enzyme activity was determined at 40°C 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 1 μmole of product per min. Digesta samples were centrifuged and the supernatant was analysed for β-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®) at 0·1% (w/v) final concentration, in 10 mM Tris–HCl pH 7·0. Activity was detected after 16 h incubation at 37°C through the Congo Red assay plate, as described in Ponte *et al*. (2004) and Mourão *et al*. (2006). Because of 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 by the azo-barley glucan method, using azo-barley glucan (Megazyme®) as substrate, following the manufacturer's 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®), according to Laemmli (1970). After electrophoresis, polypeptides were renatured by subjecting the gel to 4 washes of 30 min in 100 mM sodium succinate, pH 6·3, containing 10 mM $CaCl_2$ and 1 mM DTT. The gel was incubated overnight at 37°C 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, MO, USA) for 3 h at 37°C 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, USA) whose cup was maintained at 24°C.

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 $P < 0{\cdot}05$.

RESULTS AND DISCUSSION

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 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 1a). Linker sequences rich in hydroxyl amino acids 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 1(b), demonstrate that incubation of Lic26-Cel5-Cbm11, Lic26-Cel5, Lic26 and Cel5 with pancreatic proteases for a 3-h 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 suggest 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 1(c), demonstrate 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 into 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.

Bird performance

It remains unknown whether 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 by SDS-PAGE analysis, as exemplified in Figure 1(c). 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 d 28 to 56. The mortality rate during the experiment was low (6·2%) and was not related to 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 rain-fed 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 2, confirm 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 (Table 2) and it is clear that fibre remains the main organic component of the pasture.

Table 2. *Chemical composition of the legume-based pastures used by the free-range broilers in this experiment (g/kg DM)*

	T. subterraneum	*T. repens*
Dry matter	146·0	195·6
Crude protein	216·6	223·5
Ether extract	20·5	21·4
Crude fibre	226·6	190·9
NDF	400·2	401·5

The evolution of body weights and weight gains, total feed intake and feed conversion ratios throughout the experiment are summarised in Table 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, 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 (Table 4). 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 intakes of non-digestible material and an increase in the microbial activity in the hindgut (Brenes *et al*., 2002). Therefore, the results suggested that enzyme supplementation had no influence on 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*.

Considering the theoretically 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 as expected for the genotype RedBro Cou Nu × RedBro M (2079 g of BW at d 56; *Hubbard ISA Management Manual*). Not surprisingly, however, feed:gain ratios were considerably higher than expected

Table 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 (TsP) or T. repens (TrP) based pastures*

	TrP		*TsP*			Significance		
	No enzyme	Enzyme	No enzyme	Enzyme	SEM	*P*	*E*	*P* × *E*
Body weight (g)								
28 d	845·5	847·5	839	844·8	7·36	NS	NS	NS
35 d	1142·5	1135·3	1099·0	1135·8	12·81	NS	NS	NS
42 d	1556·8	1519·3	1499·3	1540·5	25·64	NS	NS	NS
49 d	1825·3	1819·3	1818·7	1865·3	35·28	NS	NS	NS
56 d	1978·8	1977·5	1989·7	2050·0	42·34	NS	NS	NS
Weight gain (g)								
28 to 35 d	297·0	287·8	260·3	291·0	9·18	NS	NS	
35 to 42 d	414·3	384·3	400·3	404·8	16·29	NS	NS	NS
42 to 49 d	268·8	300·0	319·3	325·0	37·88	NS	NS	NS
49 to 56 d	153·5	158·5	170·7	184·5	24·84	NS	NS	NS
28 to 56 d	1133·3	1130·0	1150·7	1205·3	41·01	NS	NS	NS
Feed intake[1] (g)								
28 to 56 d	3725·0	3761·0	3744·0	4000·8	109·09	NS	NS	NS
Feed:gain[1] ratio								
28 to 56 d	3·396	3·381	3·273	3·439	0·1690	NS	NS	NS

[1]Feed intake and feed conversion are relative to the cereal-based feed.

Table 4. *Relative weight and length of GI tract of free-range broilers fed on a barley-based feed foraging in a Trifolium subterraneum (TsP) based pasture*

	*Ts*P			
	No enzyme	Enzyme	SEM	Significance
Relative weight (g/100 g 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/100 g 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

for this genotype (expected value 2·1 to 2·2 at d 56), suggesting that the birds maintain growth rate at inappropriate temperatures and humidity by increasing feed intakes. This trial was carried out 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 given in the *Hubbard ISA Management Manual* may also result from the lower energetic concentration of the barley-based feed used in this study, 12·12 MJ EMA/kg, when compared with the recommended 13·38 MJ EMA/kg as specified in the management manual.

Forage intakes were determined by evaluating the proportion of pasture and cereal-based feed found in the crops of killed birds at the end of the experiment. The data revealed that grass biomass represents between 5·3 and 6·4% on a DM basis or 17·3 to 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 d 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*. (2008) in a similar experiment.

Meat physical properties

The influence of enzyme supplementation and pasture type on some important variables of the overall quality of poultry meat, such as carcase yield, meat pH and skin colour were investigated. The data, presented in Table 5, showed that neither enzyme supplementation nor pasture type influenced carcase 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*. 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

Table 5. *Carcase yield (%), pH and breast skin colour of free-range broilers fed on a barley-based feed foraging in Trifolium subterraneum (TsP) or T. repens (TrP) based pastures. Skin colour was expressed in the CIELAB dimensions of lightness (L), redness (a*) and yellowness (b*)*

	*Tr*P		*Ts*P			Significance		
	No enzyme	Enzyme	No enzyme	Enzyme	SEM	*P*	*E*	*P* × *E*
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 colour								
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

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 5. The data suggest that the interaction of enzyme and pasture type had no influence on meat colour 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 r carcase 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 carcases with yellow tones. Overall, the carcases 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 content of carotenoid pigments. Taken together, the data suggest that enzyme supplementation of free-range pastured broilers contributes to improving the pigmentation of the carcase, especially with pink and red tones. In addition, the botanical composition of pastures may affect their capacity to change carcase colours, with *T. repens*-based pastures being more effective in improving meat yellowness than *T. subterraneum* pastures.

Recombinant *β*-glucanase stability *in vivo*

It was anticipated that the introduction of microbial recombinant cellulases in a barley-based feed for pastured broilers might contribute to reducing the anti-nutritive properties associated with the ingestion of barley *β*-glucans. The data presented here suggest that the exogenous bi-modular enzyme had no effect on the performance of free-range broilers of a slow-growing genotype from d 28 to 56. It is recognised 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 to 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 (Svihus *et al.*, 1997; Fuente *et al.*, 1998), although it remains unknown if one or the conjunction of both these factors may operate in this case. In addition, whether pasture consumption can 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 d 1 of growth may limit the effectiveness of feed enzymes (Rosen, 2001; Bedford, 2002).

Following from 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

Table 6. *Qualitative detection of β-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 (TrP) or T. subterraneum (TsP) based pastures*

	Qualitative β-glucanase activity[1]			
Treatment	Crop	Gizzard	Duodenum	Caecum
*Tr*P				
Enzyme	+/+/+/+/+/+/+/+	+/+/+/+/−/+/+/+	+/+/+/+/−/+/+/+	+/+/+/+/+/+/+/+
No enzyme	+/−/+/−/+/−/+/+	+/−/+/−/−/−/−/−	+/−/+/−/−/−/+/+	+/+/+/+/+/+/+/+
*Ts*P				
Enzyme	+/+/+/+/+/+/+/+	+/−/−/+/−/+/−/+	+/+/−/+/+/−/+/−	+/+/+/+/+/+/+/+
No enzyme	+/+/+/+/+/−/+/+	−/−/+/−/+/−/+/+	+/+/−/−/+/−/+/−	+/+/+/+/+/+/+/+

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

T. subterraneum pasture were tested for β-glucanase activity, using the Congo Red plate assay. The results (Table 6) demonstrate 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 *Ct*Lic26A-Cel5E truncated derivatives to proteolysis. Therefore, a considerable percentage of the exogenous enzyme resists 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 β-glucanase activity, particularly in the crop; only 4 birds out of the 16 analysed did not present detectable β-glucanase activity in the crop. However, the size of the halos corresponding to β-glucanase crop activity of non-supplemented animals was suggested to be sensibly smaller, as it can be observed in Figure 2(b). Together, these data suggest that there is considerable endogenous β-glucanase activity in the GI tract of the free-range broilers, which may have originated in enzymes present in the cereal feed (Jeraci and Lewis, 1989; Bengtsson *et al.*, 1990; Grosjean *et al.*, 1999) or in symbiotic crop microflora. To test these possibilities, β-glucanase 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 β-glucanase activity, suggesting that feed endogenous β-glucanase are of barley origin. β-Glucanase activity was quantified in supplemented and non-supplemented feed using azo-barley glucan as the substrate. The data, presented in Figure 3(a), confirm that non-supplemented feed presents considerable levels of β-glucanase activity although the quantified levels were 8 times lower than the ones observed for supplemented feed. In addition, crop β-glucanase activity of non-supplemented birds was only 4 times lower than

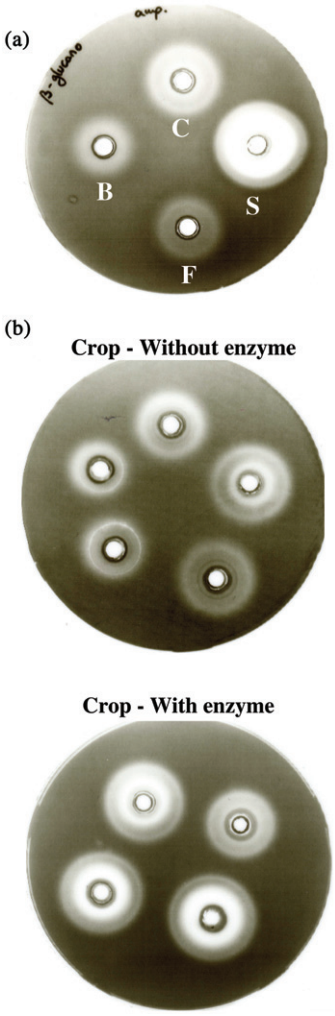

Figure 2. *Detection of β-glucanase activity in the feed (Panel A) and crop contents (Panel B) of free-range broilers fed on a barley-based diet supplemented or not supplemented with a microbial β-glucanase. Abbreviations: F = feed (without enzyme); SF = feed supplemented with the Clostridial enzyme; B = barley; Cr = crop content of a supplemented bird.*

in supplemented animals. Since Lic26-Cel5 is resistant to proteolytic inactivation, it is suggested that a fraction of the identified crop β-glucanase activity has potentially a microbial origin (Figure 3b). As a result of the described

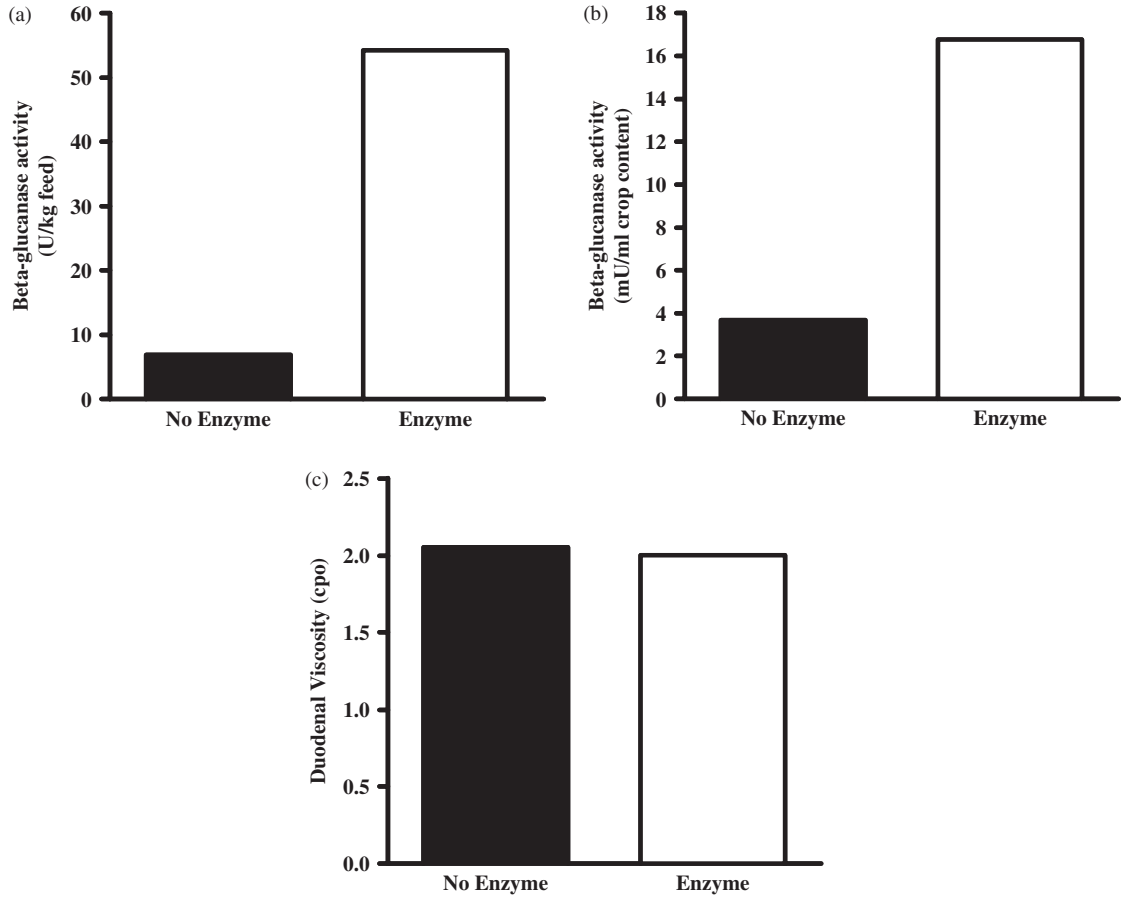

Figure 3. *Viscosity and enzyme activities of barley-based feed and duodenal contents. Panel A presents the β-glucanase activity of the barley-based feed supplemented and not supplemented with the recombinant β-glucanase* Ct*Lic26A-Cel5E. The corresponding β-glucanase activity in the crop contents of 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).*

endogenous *β*-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(c). This is not completely unexpected and suggests that the residual endogenous *β*-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 *β*-glucanase is to decrease the degree of polymerisation of soluble glucans, by randomly cleaving glycosidic bonds in the xylan backbone (Fengler and Marquardt, 1988; Bedford and Morgan, 1996). Therefore, it is possible that the moderate levels of *β*-glucanase activity observed in non-supplemented animals are responsible for decreasing the degree of polymerisation of the soluble glucans to an extent that can significantly decrease digesta viscosity.

To analyse the potential changes in the molecular architecture of the endogenous and recombinant *β*-glucanases, during passage through the GI tract, digesta samples from birds supplemented and not supplemented with the exogenous enzyme were subjected to zymogram analysis. The data (Figure 4) demonstrated that Lic26-Cel5 is prone to proteolytic cleavage in the bird GI tract, which occurs mainly in the crop and gizzard (Figure 4b, c). In Figure 4(c), it is shown that, in 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 to 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 on 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 to 80 kDa enzyme with *β*-glucanase activity in the cereal-based diet (Figure 4a). In addition, zymogram analysis of crop proteins from non-supplemented animals suggests the presence of a range of *β*-glucanases with sizes ranging from 30 to 90 kDa (Figure 4b). The variety of enzymes displaying *β*-glucanase

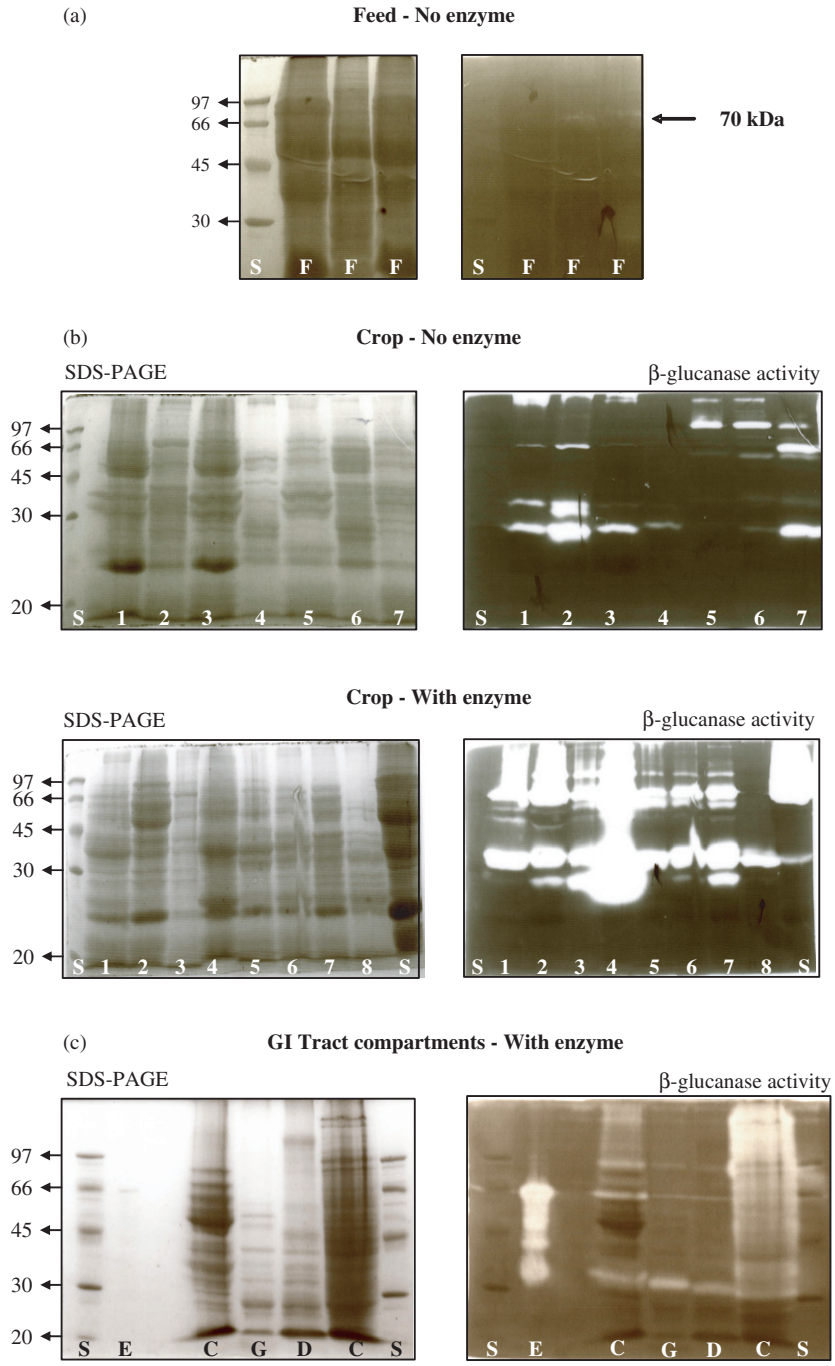

Figure 4. *Zymogram analysis of un-supplemented feed (Panel A), crop samples collected from birds supplemented or not with β-glucanase* Ct*Lic26A-Cel5E (Panel B) and digesta samples collected from various regions of the GI tract of birds supplemented with β-glucanase* Ct*Lic26A-Cel5E (Panel C). Abbreviations: F = feed (without enzyme); St = low molecular weight protein standards; SF = supplemented feed; E = enzyme; Cr = crop; G = gizzard; D = duodenum; C = caecum.*

activity identified in the crop of non-supplemented animals suggests a microbial origin.

Taken together, the results presented here suggest that in free-range pastured broilers of a slow-growing genotype and at later stages of growth the presence of β-glucanase activity expressed by crop microflora or from barley enzymes can contribute to depolymerise a significant proportion of the anti-nutritive β-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 in improving bird performance, possibly because of endogenous cellulase activity. Whether functional β-glucanases are expressed by most barley varieties or are restricted to specific samples of this cereal remains to be established. In addition, it is possible that the crop β-glucanase activity has both a plant and a microbial origin, which is only well established at later stages of the animals' growth.

CONCLUSIONS

It is well established that plant cell wall hydrolases can improve the nutritive value of cereals for simple stomach animals. Here, we showed that an individual recombinant bi-modular cellulase from *C. thermocellum* is unable to improve the nutritive value of a barley-based feed given *ad libitum* to pastured broilers of a slow-growing genotype. The data suggest that, although the enzyme suffers proteolysis in 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 β-glucanase 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 β-glucans present in barley-based diets.

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