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Review

Effects of partial substitution of grain by agroindustrial byproducts and sunflower seed supplementation in beef haylage-based finisher diets on growth, *in vitro* methane production and carcass and meat quality

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ABSTRACT

Thirty-two bulls were assigned to four total mixed biodiverse haylage-based diets to evaluate the effects of partial substitution of grains by agroindustrial byproducts, sunflower seeds (SS) supplementation and haylage level on growth, *in vitro* methane production and carcass and meat quality. Dietary treatments included a grain-based diet with 30% grain and haylage:concentrate ratio (H:C) of 60:40 (DM basis) (MCe); a by-product-based diet where 50% of the grain was substituted for by-products (H:C, 60:40) (MBp); a byproducts diet with 10% sunflower seed and 90% (DM) MBp (H:C, 54:46) (MBpSS); and a byproducts, SS diet with increased haylage (H:C, 67.5:32.5) (HBpSS). Dry matter intake and growth rate were lower in HBpSS, but feed conversion ratio was unaffected by diet. *In vitro* methane emissions were reduced by SS. Meat colour and shear force were similar among diets. Lipid oxidation in cooked meat was reduced and fatty acid composition was improved with SS. Biodiverse haylage-based diets may be a viable option for finishing bulls.

1. Introduction

The sustainability of beef production systems is a major concern for the different players in society, including producers, consumers, researchers and government decision-makers. In the south of Portugal, beef production has been traditionally associated with cereal crops, in extensive systems based on natural or sown pastures, complemented with crop residues and forages mainly in dry periods (Araújo et al., 2014). Over the last decades, some specialisation of production systems has occurred, but the feeding of cow-calf herds continues to be based on local forage resources, with low incorporation of external inputs. Calves are traditionally weaned at about 6–7 months of age and after that they are frequently confined and fed diets based on commercial concentrates until slaughter, that generally occurs up to 18 months.

The sustainability of beef production systems in general, and the finishing of young bulls in particular, could be improved by increasing the use of locally produced high-quality forages and reducing the incorporation of cereals and oleaginous meals in diets (Salami et al., 2019), in such a way that growth performance is not negatively affected. Diets based on high-quality forages can allow production performances similar to concentrate-based diets and improve meat fatty acid (FA) composition (Santos-Silva et al., 2020). Moreover, the proportion of cereals in concentrates for ruminants may be partially replaced by agroindustrial by-products, such as dehydrated sugar beet or citrus pulps and soybean hulls, without negative impacts on animal productivity or meat quality (Mueller, Blalock, & Pritchard, 2011; Santos-Silva et al., 2020; Simitzis & Deligeorgis, 2018). This promotes circularity and more rational use of available feed resources, leaving cereals to humans and monogastric animals. However, due to their high fibre content, high-forage diets have potential to enhance digestive methane (CH_4) emissions and reduce efficiency of ingested energy utilization (Johnson & Johnson, 1995). Several nutritional strategies can be adopted to

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reduce digestive CH_4 emissions by ruminants, including lipid supplementation (Hristov et al., 2013). Dietary lipid supplementation is also the main factor affecting the FA composition of ruminant meat (Mapiye et al., 2012) and supplementing high-forage diets with unsaturated lipid sources can increase deposition of healthy biohydrogenation intermediates (BI) fatty acids, vaccenic acid (t11–18:1) and rumenic acid (c9,t11–18:2) in meat (Bessa, Alves, & Santos-Silva, 2015). Sunflower seed contains more than 40% crude fat and can be an effective and practical way under commercial conditions to supplement lipids in cattle diets (Beauchemin, McGinn, & Petit, 2007; He, Mir, Beauchemin, Ivan, & Mir, 2005), without negatively effecting rumen metabolism (Dhiman et al., 2000).

Thus, we hypothesized that when using basal diets based on high-quality forages (≥60%), the partial replacement of cereals by agro-industrial by-products and the inclusion of sunflower seed as a lipid source will maintain animal production performance and increase the content of health-promoting FA in meat. Furthermore, we hypothesized that reducing cereals and increasing forage incorporation in ruminant diets would increase digestive CH_4 emissions but inclusion of sunflower seeds could counteract this effect. Thus, in the present trial, four high-forage mixed diets, differing in cereal, sunflower seed and forage proportions, were fed to crossbred calves to evaluate animal performance, *in vitro* digestive methanogenesis and carcass and meat quality.

2. Material and methods

2.1. Animals, treatments, management, slaughter and sample collection

This experiment was approved by the Animal Care Commission of the Instituto Nacional de Investigação Agrária e Veterinária I.P. (INIAV) (Proc. 01/2019), in accordance with European Union regulations for the use of production animals in experiments (EU, 2010). Thirty-two Charolais × Alentejana crossbred bull calves, born in a commercial herd in southern Portugal (Elvas, Alentejo) were used. Calves were raised by their dams under extensive grazing conditions until weaned between 6 and 7 months of age. After weaning, calves were housed together and fed with concentrate and hay *ad libitum* until transported to INIAV-Fonte Boa facilities. Upon arrival, average age and live weight (LW) were 204 ± 27.6 days and 284 ± 36.2 kg. The 32 calves were randomly assigned in pairs to 16 groups and each group to an outdoor pen with a concrete and no bedding. The 16 pens were distributed along a covered central corridor, with eight pens on either side. Pens on one side were 25 m^2 and and the other 60 m^2. All the pens had automatic waters and 3 m stainless steel feed troughs.

Four mixed diets (MD) were formulated and randomly assigned to 16 pens (4 pens per diet), blocked by the 2 pen sizes. All diets were based on high quality haylage, obtained from a biodiverse mixture of grass and trifollium annual species (Speedmix, Fertiprado, Vaiamonte, Portugal). The haylage was produced in April 2020, and had a pH of 4.03, 17.5% crude protein on a dry matter (DM basis), 40.0% NDF (DM), 54.3% soluble N, 2.4% ammonia N and 10.42 MJ/kg DM of digestible energy. Two diets (MCe, MBp) included 600 g/kg DM haylage and 400 g/kg DM concentrate (H:C, 60:40). In the MCe diet, the concentrate was formulated using corn, wheat and barley grains as the main energy source and in the MBp diet, 50% of the grains in the concentrate were replaced by an equivalent proportion of agroindustrial by-products (citrus and dehydrated beet pulp and soybean hulls). The third diet (MBpSS), included 90% of DM as MBp and 10% DM as whole sunflower seed (SS), with a H:C of 54:46. The fourth diet (HBpSS) was similar to MBpSS but with a higher proportion of, with a H:C of 67.5:32.5. The chemical composition of the MD are shown in Table 1.

Diets were formulated to be isonitrogeneous, with 160 g/kg DM of protein. The concentrates were prepared in the Feed Compound Unit of INIAV-Fonte Boa. The MD were prepared every day using a stationary mixer (Mammut, Gurten, Austria). In addition to the MD, the animals received chopped oat hay (particle size <10 cm), up to a maximum of 20% of the total wet weight of MD offered. During the experiment, the feeds were offered *ad libitum* and were distributed once a day at about 10:00 am, considering 10% refusals. The amounts of the diets offered and refused in each pen were daily recorded. Sub-samples of the MD, haylage and oats hay were collected weekly and frozen. Monthly, the sub-samples were pooled, and the composite samples were used to determine the chemical composition of the feeds, which are presented in Table 1.

The period for adaptation to the diets and experimental conditions was of 21 days. During the experiment the animals were individually weighed every 14 days. The pens were washed every day to maintain good hygienic conditions and remove manure.

When commercial slaughter weight was attained (500 to 600 kg LW), the animals were transported to an official slaughterhouse 10 km away,

Table 1
Ingredients, chemical composition and fatty acid (FA) profile of the diets.

Item	Diets				Oats hay
	MCe[1]	MBp[2]	MBpSS[3]	HBpSS[4]	
Ingredients, g/kg dry matter (DM)					
Corn	101.0	52.0	46.3	29.5	
Wheat	100.0	48.0	46.1	29.7	
Barley	100.0	48.0	46.1	29.7	
Dehydrated citrus pulp	–	51.0	46.1	29.1	
Dehydrated sugar beet pulp	–	48.0	46.1	29.0	
Soybean hulls	–	48.0	46.1	29.0	
Soybean meal	20.0	32.0	30.6	–	
Sunflower meal	30.0	24.0	3.6	–	
Calcium carbonate	13.0	13.0	13.0	13.0	
Dicalcium phosphate	9.0	9.0	9.0	9.0	
Sodium bicarbonate	20.0	20.0	20.0	20.0	
Salt	4.0	4.0	4.0	4.0	
Premix [5]	3.0	3.0	3.0	3.0	
Sunflower seed	–	–	100.0	100.0	
Haylage	600.0	600.0	540.0	675.0	
Chemical composition					
DM[2](%)	50.5	50.5	52.9	48.6	88.8
Crude Protein (% DM)	15.9	16.6	16.7	15.3	7.15
Ether extract (% DM)	2.19	2.08	6.04	6.15	1.32
Starch (% DM)	18.4	10.1	8.87	5.29	
Sugar (% DM)	3.44	5.18	4.51	3.60	
NDF (% DM)	32.3	35.1	33.5	35.5	59.2
ADF (% DM)	23.4	25.7	24.4	26.7	39.0
ADL (% DM)	3.01	3.04	3.46	3.42	5.33
Total FA[6] (% DM)	1.24	0.95	2.99	2.98	
ME (kJ)	9.83	9.70	10.47	9.61	6.26
Total phenol content (g GAE/kg DM)[7]	8.93	9.42	8.61	10.2	12.2
α-tocopherol (μg/g DM)	38.8	27.1	47.4	50.8	37.0
β-carotene (mg/g DM)	0.72	0.73	0.94	1.18	1.30
Fatty acid profile, g/kg FA[6]					
14:0	16	23	2	4	
16:0	189	217	103	107	
18:0	24	32	34	34	
*c*9–18:1	148	140	206	268	
18:2n-6	309	245	580	492	
18:3n-3	314	343	75	95	

[1] 60% DM of haylage and 40% DM of concentrate with cereals.
[2] 60% DM of haylage and 40% DM of concentrate with cereals + by-products.
[3] 54% DM of haylage, 36% DM of concentrate with cereals + by-products and 10% DM of sunflower seed.
[4] 67.5% DM of haylage, 22.5% DM of concentrate with cereals + by-products and 10% DM of sunflower seed.
[5] Premix composition/kg vitamins: A, 4000000 UI, D3, 1,000,000 UI, E, 15000 mg and B1, 1000 mg; trace elements zinc, 20,000 mg, copper, 1000 mg, manganese, 15,000 mg, iodine, 250 mg, cobalt 300 mg, selenium, 100 mg, (BHT) (E321) 100 mg and magnesium oxide (excipient) 75,000 mg.
[6] Fatty acid.
[7] Gallic acid equivalents.

after being weighed. To allow most animals to attain the target weights, bulls were slaughtered on two dates 14 days a part, corresponding to 140 and 154 days of trial. Animals slaughtered on the same day were distributed equally between treatments. The animals had free access to feed and water up to 2 h before slaughter. Upon arrival at the slaughterhouse, the animals sacrificed as soon as possible by exsanguination, after being stunned using a penetrating captive bolt in compliance with European standards for the protection of animals at the time of killing (EU, 2009). Electric stimulation was applied during bleeding.

About 30 min after slaughter, 1 L of whole rumen content was individually collected and stored in a plastic container that was maintained in a water bath at 38 °C during transportation to the INIAV-Fonte Boa laboratory. After dressing, the carcasses were graded for conformation and fattening according to the SEUROP grading scheme (Comission Regulation (EC), 2008) and weighed. The carcasses were split along the spine and chilled in a refrigeration chamber at 0 °C for 24 h. Forty-eight hours after slaughter, the half carcasses were separated into anterior and posterior sections at the 9th thoracic vertebra and transported to an industrial processing unit. The colour of leg subcutaneous fat (ScF) was evaluated over the *Semimembranosus* muscle, and over the proximal portion of the 12th thoracic vertebra of left carcass sides. Samples of ScF and *Longissimus thoracis* (LT) muscle were collected between the 9th and 12th thoracic vertebra from all carcasses. The samples were vacuum-packed and transported immediately, under refrigeration conditions, to INIAV-Fonte Boa facilities. Subcutaneous fat samples were immediately processed for FA analysis. The LT samples were kept under refrigeration until 72 h post-slaughter, then divided into 4 sub-samples of approximately 2 cm thickness. After the removal of epimysium, two sub-samples of LT were minced in a food processor (Moulinex-123 A320R1, Group SEB Portugal Lda, Lisbon, Portugal) (3 × 5 s) and vacuum packed then stored at −20 °C until chemical composition and pH determinations. The other two sub-samples were used to evaluate colour, lipid stability and shear force at days 3 and 14 after slaughter. During storage, meat was preserved under vacuum and maintained at 2 °C in the dark until 14 days after slaughter. Colour parameters were determined after 60 min blooming. After colour evaluation, a small portion of LT muscle was removed (± 25 g), vacuum-packed and stored at −80 °C until lipid oxidation analysis. The remaining LT samples were vacuum-packed and stored at −20 °C until cooking loss and shear force determinations.

2.2. Feed analysis

The feeds used in the experiment were analysed for DM, crude protein (CP) and ether extract (EE) according to ISO6496 (1999), ISO5983 (1997) and ISO6492 (1999), respectively. For starch and sugar determinations the method described by Clegg (1956) was used. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were measured according to Van Soest, Robertson, and Lewis (1991). The NDF was quantified with sodium sulfite and without α-amylase and expressed with the ash residue. Feed FA were determined by direct transesterification of feed lipids and using nonadecanoic acid (19:0) as the internal standard according to the procedures described by Sukhija and Palmquist (1988) and FA methyl esters (FAME) were analysed using a gas chromatograph (HP6890, Agilent, Avondale, PA, USA), equipped with a flame-ionisation detector and a fused silica column (SP Omegawax™ 250, Supelco, Bellefonte, PA, USA) with 30 m of length, 0.25 mm internal diameter and 0.25 μm film thickness. The conditions of chromatography were as described by Francisco et al. (2016).

Total phenols were determined in an extract prepared according to Julkunen-Tiitto (1985) and Makkar, Gamble, and Becker (1999) using the Folin-Ciocalteu's assay according to Falleh et al. (2008). Gallic acid was used as a standard and results were expressed as gallic acid equivalents (GAE). Tocopherol and ß-carotene extracts were prepared according to Ball (1992), with slight modifications. The analyses were performed according to Prates, Quaresma, Bessa, Fontes, and Alfaia (2006), using a Dionex Ultimate 3000 uHPLC (Thermo Fisher Scientific) and 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). The α-tocopherol was identified using fluorescence detection (excitation wavelength of 295 nm and emission wavelength of 325 nm). ß-carotene was determined simultaneously using UV–Vis photodiode array detection at 450 nm. The α-tocopherol and ß-carotene contents were calculated using an external calibration curve using pure α-tocopherol (Calbiochem, USA) and ß-carotene (Sigma Chemical Co., St. Louis, MO, USA).

2.3. Determination of in vitro methane emission

One L of mixed rumen content of each animal was collected in the slaughterhouse 30 min after slaughter and maintained in closed plastic bottles immersed in tap water (38 °C) during transportation to INIAV-Fonte Boa laboratory. The rumen samples were processed 90 min after slaughter. After filtration through 4 layers of cheesecloth, a sample of the liquid fraction (rumen liquor) was used for pH determination using a Metrohm 744 pH meter (Metrohm AG, Switzerland) and another sample was used for the *in vitro* determination of CH_4 emissions.

Methane production was measured using fully automated gas production equipment ($ANKOM_{RF}$ Gas Production System, ANKOM Technology, NY, USA). Inocula used were prepared by mixing the rumen liquor of each animal with an anaerobic buffer/mineral solution in a 1:2 ratio (*v*/v) (Menke et al., 1979). Diet samples (1 g DM) were weighed in duplicate into 250 mL glass bottles containing 90 mL buffered rumen solution and 2 mL of a reducing solution (Menke et al., 1979) for 48 h in a shaking water bath at 39 °C. The gas pressure and temperature were automatically recorded during the incubation period in a database using dedicated software (BacVis). The gas pressure was converted to moles of gas using the ideal gas law and converted to mL of gas produced using Avogrado's law. At the end of each incubation cycle, gas samples were collected from the headspace of each bottle and analysed for CH_4 by GC (HP6890A, Agilent, Avondale, PA, USA) using a capillary column (TG-Bond Q 30 m × 0.53 mm × 20 μm, Thermo Scientific) and flame ionization detection. The GC column temperature was held at 150 °C and the injector and detector temperatures were 200 °C and 220 °C, respectively. For each run, 100 μL of gas was injection, using a syringe Pressure lock series A-2 (1 mL) (Supelco, Bellefonte, PA, USA), considering 5 replicates per sample.

For volatile fatty acids (VFA) the stained rumen fluid samples, were prepared by adding 170 μL of orthophosphoric acid solution (25/100, *v*/v) and 130 μL of internal standard (C 5:0 at 50 mmol/L) and then centrifuge at 15000 ×*g* for 15 min. The supernatant was analysed for using a gas chromatograph (HP7683, Agilent, Avondale, PA, USA) equipped with a flame ionisation detector and fused capillary column (Nukol, Supelco, Bellefonte, PA, USA) with 30 m, 0.25 mm internal diameter and 0.25 m film thickness. Helium was the carrier gas and the split ratio was 1:50. The injector and detector temperatures were 250 °C and 280 °C, respectively. The column operated isothermally at 180 °C for 10 min. Volatile FA were quantified using calibration curves, which were prepared for each VFA at concentrations ranging from 0.2 to 30 mmol/L and using C 5:0 as internal standard at 50 mmol/L.

2.4. Meat and subcutaneous fat

Longissimus thoracis pH was measured 72 h after slaughter, in a suspension of 5 g of minced meat in 50 mL of potassium chloride 0.1 M, using a Metrohm 744 pH meter (Metrohm AG, Switzerland) equipped with a combined glass electrode and according to ISO2917 (1999). Dry matter and crude protein of LT were determined according to ISO1442 (1997) and (ISO5983, 1997), respectively.

Lipid extractions from freeze-dried LT and ScF were done according to Folch, Lees, and Stanley (1957). Lipids extracted from meat and ScF

were methylated using sodium methoxide in anhydrous methanol (0.5 M) for 30 min at 50 °C, followed by hydrochloric acid in methanol (1.25 M) for 10 min at 50 °C. For FA quantification, nonadecanoic acid (19:0) was used as the internal standard (Alves, Raundrup, Cabo, Bessa, & Almeida, 2015). Methyl ester composition was analysed using a Shimadzu GC 2010-Plus gas chromatograph (Shimadzu, Kyoto, Japan), equipped with a flame-ionisation detector and fused silica capillary column (SP2560 100 m × 0.25 mm internal diameter × 0.20 μm film thickness, Supelco Inc., Bellefonte, PA, USA). Chromatographic conditions used were as described by Francisco et al. (2015). Fatty acid methyl esters were identified by comparing the retention times with commercial standard mixtures (FAME mix 37 components from Supelco Inc.) or with published chromatograms by Alves and Bessa (2009) and Vahmani, Rolland, Gzy, and Dugan (2016). Additional identification of the FAME was achieved by electron impact mass spectrometry using a Shimadzu GC–MS QP2010 Plus (Shimadzu, Kyoto, Japan) using method described by (Alves, Francisco, Costa, Santos-Silva, & Bessa, 2017).

Lipid oxidation was measured in cooked meat from each ageing time (3 and 14 days). Measurements were taken immediately after cooking and after 3 days cold storage. Sample preparation occurred in 7 sessions, considering just one batch per session. For each ageing time, two vacuum packaged slices were cooked for 30 min at 70 °C in a water bath. Lipid oxidation was immediately measured in one slice, while other was placed on a polystyrene tray, wrapped with oxygen permeable polyvinyl chloride film and stored at 4 °C for 3 days. Lipid oxidation was assessed by measuring thiobarbituric acid reactive substances (TBARS), according to Grau, Guardiola, Boatella, Barroeta, and Cordony (2000) using a 1,1,3,3 tetraethoxypropane standard curve and results were expressed as mg of malonaldehyde (MDA)/kg of meat.

Meat colour was evaluated using a CR-400 chromometer (Konica Minolta, Japan) using a 10 mm diameter aperture, a D65 illuminant and 2° observer. The chromometer was calibrated each day using a white standard plate (D65: Y84.9, x0.3199, y 0.3359). Measurements were recorded in the CIELAB system, where L* is lightness, a* redness and b* yellowness. Colour saturation (C^*) and hue angle (H^*) were calculated as $(a^{*2} + b^{*2})^{1/2}$ and $\tan^{-1}(b^*/a^*) \times (180/\prod)$, respectively (AMSA, 2012). Colour stability during 11 days of vacuum storage (between days 3 and 14 after slaughter) was evaluated using the colour stability index (ΔE), determining the colour difference between the two measurements $\Delta E_{(14)} = ((L^*_{14} - L^*_3)^2 + (a^*_{14} - a^*_3)^2 + (b^*_{14} - b^*_3)^2)^{1/2}$ at days 3 and 14 after slaughter (Ripoll, Albertí, & Joy, 2012).

Meat cooking loss was determined according to a procedure adapted from Honikel (1998). Frozen LT samples were thawed for 24 h at 2 ± 1 °C. The epimisium was removed and samples were weighed (215 ± 5 g) before being placed in a plastic bag and immersed in water at 80 °C until attaining an internal temperature of 75 °C. Water-bath temperature was controlled using a heating system proportional to the set point (Grant Instruments Ltd., Type KA 1.5KW, Barrington Cambridge CB2 5QZ, England), which maintains the water temperature in a range of 80 ± 0.1 °C. Sample temperature was monitored using an internal type T thermocouple (Thermometer Omega RDXL4SD, Manchester, USA). This procedure was repeated in 4 sessions on different days. According to AMSA (2016), the samples were cooled for 20 h at 2 ± 1 °C and then were reweighed to determine cooking loss as a percentage of the weight before cooking. The same samples were used for shear force determination. From each sample, 8–10 cores parallel to fibre direction and with a cross section of 1 cm^2, were cut and used for texture analysis, measured by means of force *vs.* time in compression to determine peak force. Maximum shear force was determined, using a Warner-Bratzler shear attachment on a Stable Micro Systems TA.XT Plus Texture Analyser (Surrey, UK). Trigger force was of 25 g and crosshead speed during pre-test, test and post-test set were 5.0; 2.0 and 10.0 mm/s, respectively. Time, force and distance were recorded at 200 points/s and analysed with the Version 6.1.16 of Exponent software (Stable Micro Systems - Surrey, UK).

2.5. Statistical analysis

The experiment was conducted as a randomised design with pen as the experimental unit. Statistical analysis was conducted using the Proc MIXED of SAS (SAS Institute Inc., Cary, NC). Variance heterogeneity was accommodated in models when significant ($P < 0.01$), using the group option within the repeated statement of the Proc MIXED.

For the determination of the average daily weight gains (ADG) a random intercept regression model was used for the analysis of the individual weights recorded over the experiment and pen size was included as random factor. Daily intake of DM and nutrients were analysed using a model that included the diet as fixed effect and pen size as a random effect. Feed intake data were repeated measures over time from each pen. Therefore, the model used for intake also included the day of trial, considering a first order autoregressive (AR(1)) covariance structure, selected based on the Akaike information criteria (AICC).

Feed conversion ratios and feeding costs were estimated for each period of 14 days, between 2 consecutive weightings. The outliers were removed, using the Interquartile Range method, considering Q1 as 25% and Q3 as 75%, and 1.5 as adjustment factor. The results were analysed as repeated measures in the pen, using a model that included the diet as the main factor, the fourteen day measurement period as the block and the size of the pen as a random effect, considering the autoregressive (AR (1)) covariance structure.

Live slaughter weight and carcass data, ScF colour and meat chemical composition traits were analysed using a model that included the diet as a fixed effect, pen as experimental unit and the day of slaughter as a random effect. Initial LW was included in the model as a covariate for the analysis of slaughter and carcass weights. Subcutaneous fat colour traits were analysed using a model that included the diet as a fixed effect and location in the carcass as a blocking factor. The pen was the experimental unit and the day of slaughter was included in the model as a random effect. Meat colour was analysed using a model that included diet and ageing period (3 and 14 days) as fixed effects and the diet × ageing period interaction. Due to the correlated nature of the meat subsamples from each animal, the subject option within the repeated statement of SAS and the autoregressive (AR(1)) covariance structure were used. The model also included the day of slaughter and pen as random effects. A similar model was used for lipid oxidation, cooking loss and shear force with the additional random effect of the day of sample preparation. Meat tissue FA composition were analysed using a model that included the diet and tissue (LT *vs* ScF) as fixed effects and the diet × tissue interaction, which was removed from the model when not significant. Total FA content of LT was used as covariate for individual FA analysis.

Data presented were least-square means for fixed effects and interactions when significant. Statistical significance was set at $P < 0.05$ and trends toward significance at $0.05 < P < 0.10$.

3. Results

3.1. Feed intake and growth performance

The results observed for DM and nutrient intakes are presented in Table 2. The daily intake of DM was lower in HBpSS than in MBp and MCe and MBpSS presented intermediate values ($P = 0.042$). When reported on a metabolic live weight basis, the HBpSS had lower intake than other diets, averaging 91 g/$kgLW^{0.75}$. Intakes of fibrous components, NDF and ADF, were not affected by treatments with averaged 2682 ± 402.1 and 1967 ± 290.3 kg/day, respectively. However, for of ADL, the intake was higher with MBpSS ($P < 0.001$). The protein intake was lower in HBpSS than MBp and MBpSS, and MCe presented an intermediate value ($P = 0.011$). Intake of ether extract reflected the differences in diet formulas and was higher for MBpSS and HBpSS than for MCe or MBp diets ($P < 0.001$). Intake of starch was higher for MCe, followed by MBp and by MBpSS, and lower for HBpSS ($P < 0.001$).

Table 2
Effects of the diet on dry matter and nutrients intake of crossbred Charolais x Alentejana young bulls.

Intake (g/day)	Diets				*P*-value
	MCe[1]	MBp[2]	MBpSS[3]	HBpSS[4]	
Dry matter (DM)	8040 ± 700.4 ab	8258 ± 383.3 b	7798 ± 383.3 ab	7228 ± 375.9 a	0.042
DM/kgLW$^{0.75}$	91.1 ± 2.45 b	92.4 ± 1.75 b	89.1 ± 1.75 b	85.6 ± 1.75 a	0.003
Crude protein	1271 ± 112.0 ab	1362 ± 59.8 b	1276 ± 59.8 b	1112 ± 59.8 a	0.011
Ether extract	175 ± 9.9 a	171 ± 9.9 a	511 ± 24.1 b	506 ± 24.1 b	<0.001
Sugar	269 ± 17.0 a	414 ± 17.0 c	353 ± 15.6 b	260 ± 15.6 a	<0.001
Starch	1461 ± 115.0 c	821 ± 27.6 b	720 ± 27.6 b	386 ± 18.9 a	<0.001
NDF [5]	2636 ± 221.8	2944 ± 126.2	2584 ± 126.2	2564 ± 127.7	0.056
ADF [6]	1899 ± 161.3	2149 ± 92.2	1876 ± 92.2	1946 ± 95.1	0.077
ADL [7]	238 ± 14.4 a	254 ± 10.4 a	289 ± 10.8 b	250 ± 10.8 a	<0.001
ME (MJ)	78.6 ± 5.14	79.7 ± 3.21	81.4 ± 3.21	69.4 ± 5.14	0.485
Total phenols (g)	70.2 ± 4.14 b	77.7 ± 2.83 c	67.2 ± 2.83 a	73.4 ± 3.01 b	<0.001
α-tocopherol (μg)	303 ± 18.8 b	224 ± 13.0 a	367 ± 13.0 c	363 ± 14.2 c	<0.001
β-carotene (mg)	5.70 ± 0.360 a	6.07 ± 0.272 a	7.33 ± 0.272 b	8.48 ± 0.304 c	<0.001
Total FA	61.4 ± 1.83 a	70.0 ± 1.83 b	339.6 ± 20.54 d	257.7 ± 20.54 c	<0.001
14:0	1.01 ± 0.030 a	1.59 ± 0.030 c	2.93 ± 0.047 d	1.46 ± 0.030 b	<0.001
16:0	11.6 ± 0.34 a	15.2 ± 0.34 b	37.4 ± 1.17 d	28.7 ± 1.17 c	<0.001
18:0	1.47 ± 0.044 a	2.22 ± 0.044 b	12.4 ± 0.77 c	10.4 ± 0.77 c	<0.001
*c*9–18:1	9.10 ± 0.272 a	9.79 ± 0.272 a	66.4 ± 5.28 c	44.5 ± 5.28 b	<0.001
18:2n-6	18.9 ± 0.58 b	17.1 ± 0.58 a	190.2 ± 14.54 d	141.7 ± 14.54 c	<0.001
18:3n-3	19.3 ± 0.62 a	24.2 ± 0.31 b	30.6 ± 0.62 c	31.4 ± 0.96 c	<0.001

[1] 60% DM of haylage and 40% DM of concentrate with cereals.
[2] 60% DM of haylage and 40% DM of concentrate with cereals + by-products.
[3] 54% DM of haylage, 36% DM of concentrate with cereals + by-products and 10% DM of sunflower seed.
[4] 67.5% DM of haylage, 22.5% DM of concentrate with cereals + by-products and 10% DM of sunflower seed.
[5] Neutral detergent fibre.
[6] Acid detergent fibre.
[7] Lignin; values with different superscripts are significantly different ($P < 0.05$).

Intake of sugars was higher in MBp, intermediate for MBpSS and lower in MCe and HBpSS ($P < 0.001$).

Intake of total phenols was higher in MBp, followed by HBpSS and MCe and finally by MBpSS ($P < 0.001$). Intakes of α-tocopherol for MBpSS and HBpSS were 20% higher than MCe and 63% higher than in MBp ($P < 0.001$). Intake of β-carotene was higher in HBpSS, followed by MBpSS and finally by MCe and MBp ($P < 0.001$). Intake of the main FA present was affected by diet. Intakes of total FA, oleic (c9–18:1) and linoleic (18:2n-6) acids were higher for MBpSS, intermediate for HBpSS and lower for MBp and MCe that showed similar values between them ($P < 0.001$). Intake of myristic acid (14:0) was higher ($P < 0.001$) for MBpSS, followed by MBp, HBpSS and finally by MCe. Intake of palmitic acid (16:0) was higher for MBpSS, followed by HBpSS, MBp and finally by MCe ($P < 0.001$). Intakes of stearic acid (18:0) and linolenic acid (18:3n3) were higher in MBpSS and HBpSS, followed by MBp and finally by MCe ($P < 0.001$).

The growth rate, conversion ratios and production costs results are presented in Table 3. Slaughter weight was not affected by diet, averaging 495 ± 44.6 kg. Average daily weight gain (ADG) was lower ($P < 0.001$) for HBpSS (1350 g/day) than for the other diets, which presented similar results, averaging 1554 g/day. The feed conversion ratio was similar between diets when reported as fed or DM weight, averaging 10.7 ± 2.76 and 5.43 ± 1.386, respectively. The consumption of concentrate per kg LW gain was lower ($P < 0.001$) for HBpSS (2.03 kg/kg LW gain) and higher for MBpSS (2.75 kg/kg LW gain), and MCe and MBp presented intermediate values. The feeding costs reported based ib. live weight gain were estimated based on the local prices of raw materials in 2021. The feeding costs were similar for MCe and MBp and 12% lower than for MBpSS, HBpSS was intermediate ($P = 0.036$).

3.2. *In vitro* methane production

The effects of the diets on *in vitro* gas and CH_4 production after 48 h of incubation is shown in Table 4. No significant differences were observed in the CH_4 concentration in total gas, but HBpSS generated less gas than MCe and MBp and, consequently, less CH_4 was produced. The MBpSS diet generated gas and CH_4 values intermediate between MCe / MBp and HBpSS. *In vitro* production of total volatile fatty acids (VFA) was not affected by diet ($P = 0.442$).

3.3. *Carcass and meat quality traits*

Carcass quality traits and meat chemical composition are presented in Table 5. Carcass weight and dressing percentage were not affected by diets, averaging 280.4 ± 25.28 kg and 56.7 ± 1.83%, respectively. Carcasses were graded for conformation and external fatness. In all the diets, 75% of the carcasses were graded as "R" and 25% as "O" for conformation and most of the carcasses (75–100%) were graded in class "2" and the remaining in class "3". Subcutaneous fat colour and meat chemical composition were not affected by treatments.

Physical meat quality traits and oxidative stability were evaluated in LT on days 3 and 14 after slaughter. The results are presented in Table 6. Independent of diet, L^*, a^*, b^* and intensity (C^*) increased with ageing period ($P < 0.001$). However, hue-angle (H^*) values and colour stability over ageing period (ΔE) were not affected by the ageing period. Meat

Table 3
Effects of the diet on growth performance of crossbred Charolais x Alentejana young bulls.

	Diets				SEM[5]	*P*-value
	MCe[1]	MBp[2]	MBpSS[3]	HBpSS[4]		
Growth performance						
Slaughter weight (kg)	500	508	505	467	16.5	0.324
ADG[6] (g/d)	1546 b	1552 b	1563 b	1350 a	34.1	<0.001
As fed FCR[7]	10.9	11.0	10.0	11.1	0.49	0.154
DM FCR[8]	5.50	5.28	5.38	5.62	0.246	0.682
Concentrate (kg/kg live weight gain)	2.45 b	2.30 b	2.75 c	2.03 a	0.099	<0.001
Cost (€/kg weight gain)	1.55 a	1.56 a	1.77 b	1.70 ab	0.062	0.036

[1] 60% DM of haylage and 40% DM of concentrate with cereals.
[2] 60% DM of haylage and 40% DM of concentrate with cereals + by-products.
[3] 54% DM of haylage, 36% DM of concentrate with cereals + by-products and 10% DM of sunflower seed.
[4] 67.5% DM of haylage, 22.5% DM of concentrate with cereals + by-products and 10% DM of sunflower seed.
[5] Standard error of the means.
[6] Average daily weight gain.
[7] As fed feed conversion ratio.
[8] Dry matter feed conversion ratio; values with different superscripts are significantly different ($P < 0.05$).

Table 4
In vitro methane production of the diets and total volatile fatty acids (VFA) after the incubation.

	Diets				SEM[5]	*P*-value
	MCe[1]	MBp[2]	MBpSS[3]	HBpSS[4]		
Gas Production (ml/g DM[6])	208 c	179 bc	168 ab	139.4 a	12.16	0.029
Methane Concentration (%)	27.5	27.6	24.3	20.8	2.66	0.243
Methane Production (ml/g DM[6])	57.1 b	55.8 b	41.5 ab	30.4 a	6.21	0.048
Methane Production (ml/g OM[7])	64.6 b	63.2 b	47.0 ab	35.3 a	7.10	0.056
Total VFA (mmol l^{-1})	73.4	85.9	94.0	95.4	10.43	0.442

[1] 60% DM of haylage and 40% DM of concentrate with cereals.
[2] 60% DM of haylage and 40% DM of concentrate with cereals + by-products.
[3] 54% DM of haylage, 36% DM of concentrate with cereals + by-products and 10% DM of sunflower seed.
[4] 67.5% DM of haylage, 22.5% DM of concentrate with cereals + by-products and 10% DM of sunflower seed.
[5] Standard error of the means.
[6] Dry matter.
[7] Organic matter; values with different superscripts are significantly different ($P < 0.05$).

Table 5
Effects of the diet on carcass characteristics and meat chemical composition of crossbred Charolais x Alentejana young bulls.

	Diets				SEM[5]	*P*-value
	MCe[1]	MBp[2]	MBpSS[3]	HBpSS[4]		
Carcass traits						
Hot carcass weight (kg)	288.4	294.6	290.4	271.1	9.18	0.344
Dressing percentage (%)	56.7	56.9	56.4	56.7	0.70	0.975
Subcutaneous fat colour						
*L**	72.4	73.0	73.1	73.8	0.57	0.424
*a**	4.01	3.92	3.79	3.48	0.473	0.870
*b**	8.90	10.1	8.75	9.42	0.470	0.235
*C**	9.87	10.9	9.60	10.1	0.591	0.442
*H**	66.1	70.4	67.6	71.1	1.82	0.221
Meat chemical composition						
Dry matter (%)	24.3	24.3	24.8	23.6	0.35	0.166
Crude protein (%)	22.5	22.2	22.4	21.7	0.37	0.107
Crude fat (%)	2.02	2.28	2.41	2.21	0.28	0.602
pH	5.80	5.81	5.71	5.88	0.076	0.535

[1] 60% DM of haylage and 40% DM of concentrate with cereals.
[2] 60% DM of haylage and 40 % DM of concentrate with cereals + by-products.
[3] 54% DM of haylage, 36% DM of concentrate with cereals + by-products and 10% DM of sunflower seed.
[4] 67.5% DM of haylage, 22.5% DM of concentrate with cereals + by-products and 10% DM of sunflower seed.
[5] Standard error of the means. *L** - lightness; *a** - redness; *b** - yellowness; *C**- chroma; *H**– hue; ΔE - colour stability index; values with different superscripts are significantly different ($P < 0.05$).

lipid oxidation was not affected by ageing time when measured immediately after cooking ($P = 0.113$) or after 3 days of cooking ($P = 0.103$). Immediately after cooking, meat lipid oxidation was also not affected by the diet ($P = 0.235$). Conversely, on the 3rd day after cooking, meat from bulls fed MBpSS and HBpSS diets showed lower TBARS values ($P < 0.001$, 2.88 g MDA/kg meat) than those from bulls fed MCe and MBp diets (3.65 g MDA/kg meat).

Beyond meat colour and lipid oxidation, the cooking loss and shear force were also evaluated at the 3rd and 14th day after slaughter. Shear-force results were not affected by diet, but varied over ageing time ($P < 0.001$), with shear-force values 17% lower at day 14 after slaughter than when measured at day 3 after slaughter ($P < 0.001$). Meat cooking loss was not affected by diet or ageing time.

3.4. Fatty acid composition of meat lipids

The results of LT and ScF general FA composition and the FA intermediates of the ruminal biohydrogenation (BH) are presented in Tables 7 and 8, respectively. Total FA was only affected by the tissue, with average values of 65.3 ± 20.49 mg/g DM in LT and 761 ± 111.8 mg/g DM in ScF.

As expected, the location of fat deposition influenced the FA composition, having as main differences higher depositions of polyunsaturated FA (PUFA) and biohydrogenation intermediates (BI)-18:1 and BI-18:2 in ScF than in LT.

The diet did not affect the sum of even-chain saturated FA ($P = 0.096$), but 14:0 was higher in MBp and HBpSS than in MCe, and MBpSS was intermediate. Palmitic acid (16:0) was higher in MBp than in the other diets and 18:0 was higher in sunflower seed supplemented diets. Considering monounsaturated FA, *c*9–16:1 was higher in MBpSS than other diets that presented similar values, *c*9–17:1 was higher in MBp than in MBpSS and HBpSS, and MCe was intermediate and *c*9–19:1 was lower when sunflower seed was supplemented.

The supplementation of diets with sunflower seed caused a reduction of total odd-chain FA and 17:0 mainly when compared to MBp. Branched-chain FA and individual i-16:0, i-17:0, a-17:0 and i-18:0, presented a similar pattern.

Considering biohydrogenation intermediates (Table 8), most FA were higher in sunflower seed supplemented diets (MBpSS and HBpSS). The only exceptions to this general pattern were *c*13–18:1, which was not affected by diet, and *t*11,*c*15–18:2, for which the results were dependent of the depot. For the *t*11–18-1 and *c*9,*t*11–18:2, the effect of sunflower supplementation was more pronounced when the level of forage in the diet was higher (75% DM), particularly in ScF. However, the *t*10–18:1 / *t*11–18:1 ratio was not affected by diet.

4. Discussion

The diets used in the present trial were all based on high-quality haylage from biodiverse forages. The diets had the same physical form and similar NDF content and all were well accepted by the animals. Dry matter intake was 10% reduced when the level of haylage in the diets was higher, suggesting that voluntary feed intake was regulated by rumen capacity and that rumen outflow was lower with HBpSS (Forbes, 2007). Although dietary lipid supplementation can reduce DM intake (Bessa, Portugal, Mendes, & Santos-Silva, 2005; Palmquist, 1994), inclusion of 10% sunflower seed in MBpSS diets did not depress DM intake when compared to MBp. The whole sunflower seed used in this experiment was an effective and practical method of lipid supplementation, confirming previous reports (Beauchemin et al., 2007). However, a better solution would be the use of other unsaturated lipid sources, preferencially less expensive and not consumed by humans, such as grape seed or full fat corn germ.

The inclusion up to 15% of agroindustrial by-products in the diet, replacing 50% of the cereals in the concentrate, did not affect DM intake, in accordance with results reported for cattle (Ahooei, Foroughi, Tahmasbi, Shahdadi, & Vakili, 2011) or lambs (Francisco et al., 2020; Lanza, Priolo, Biondi, Bella, & Salem, 2001).

The growth rates observed in this experiment were superior to other reports for Alentejana purebred young bulls raised using intensive finishing systems (Carolino, 2006; Santos-Silva et al., 2020), but lower than those observed by our team in a trial with crossbred Limousine × Alentejana young-bulls fed similar diets as used in this experiment (1774 to 1895 g/day) (Santos-Silva, 2020). The lower ADG for HBpSS likely reflects the lower energy density, which was not offset by increased DM consumption. Feed conversion ratio (FCR) was not affected by

Table 6
Effects of the diet and of the days of ageing on colour parameters, on colour and lipid stability and cooking loss and shear force of m. *Longissimus thoracis* of crossbred Charolais x Alentejana young bulls.

	Diet				Days of ageing		*P*-value	
	MCe[1]	MBp[2]	MBpSS[3]	HBpSS[4]	3	14	Diet	Day
*L**	41.8 1.15	43.1 1.15	44.1 0.41	42.2 1.11	42.0 0.53	43.6 0.53	0.135	<0.001
*a**	20.0 1.12	19.1 1.12	21.0 0.95	19.5 1.20	18.9 0.96	20.9 0.96	0.110	<0.001
*b**	12.5 0.81	12.3 0.81	13.5 0.45	12.2 0.81	11.8 0.53	13.4 0.53	0.057	<0.001
*C**	23.6 1.38	22.6 1.38	24.9 0.97	22.9 1.38	22.2 1.04	24.8 1.04	0.068	<0.001
*H**	31.9 0.67	32.8 0.67	32.9 0.67	31.8 0.67	31.9 0.43	32.8 0.43	0.507	0.133
ΔE	4.89 0.709	5.48 0.709	5.52 0.709	5.33 0.709	–	–	0.916	–
Cooking loss (%)	30.7 0.68	31.2 0.68	29.7 0.68	30.7 0.68	30.5 0.59	30.7 0.59	0.336	0.786
Shear force (N/cm^2)	45.3 2.71	38.6 2.53	45.5 2.55	42.2 2.71	42.6 1.42	30.6 1.42	0.215	<0.001
0 days after cooking	0.12 0.012	0.14 0.013	0.12 0.013	0.11 0.013	0.13 0.011	0.11 0.012	0.235	0.180
3 days after cooking	3.47b 0.138	3.82b 0.138	2.87a 0.140	2.88a 0.142	3.02 0.117	3.48 0.105	<0.001	0.103

[1] 60% DM of haylage and 40% DM of concentrate with cereals.
[2] 60% DM of haylage and 40% DM of concentrate with cereals + by-products.
[3] 54% DM of haylage, 36 % DM of concentrate with cereals + by-products and 10% DM of sunflower seed.
[4] 67.5% DM of haylage, 22.5% DM of concentrate with cereals + by-products and 10% DM of sunflower seed; *L** - lightness; *a** - redness; *b** - yellowness; *C**- chroma; *H**– hue; ΔE - colour stability index; values with different superscripts are significantly different ($P < 0.05$).

treatments presenting values ranging from 5.28 to 5.62 when reported based on DM, which was lower than others observed in Alentejana young-bulls fed with diets based on concentrate or forage (Santos-Silva et al., 2020) or crossbred Limousine × Alentejana young-bulls fed similar diets (Santos-Silva, 2020). The feeding costs per kg of live weight gain were determined based on local prices of raw materials for concentrate and the market price of haylage in 2021. Feeding costs were higher when using sunflower seed as a supplement (13% higher when MBpSS is compared to MBp), resulting from the difference in diet costs (0.1775 € / kg *vs* 0.1407 € / kg). The feeding costs obtained here were lower than those obtained by our team for crossbred Limousine × Alentejana young-bulls, raised in the same weight range and management conditions but fed with a conventional concentrate-based diet (1.77 €/kg weight gain (prices of 2021)) (Santos-Silva, 2020). This suggests that the feeding costs of diets based on high-quality haylage can be competitive with those of conventional diets. Another important objective for using high-quality forage-based diets for young bulls in the finishing phase is to minimise the external inputs by reducing concentrate intake. In the present experiment, the average consumption of concentrate per kg of weight gain ranged among 2.03 and 2.75 kg for diets HBpS and MBpS respectively. In a previous trial with Limousin × Alentejana crossbred young-bulls fed a conventional diet, we observed values of concentrate consumption per kg of gain of 5.1 ± 0.29 kg suggesting that when high quality haylage-based diets are used, concentrate intake can be reduced by 45–60%, maintaining high levels of productivity.The reduction in the consumption of cereals may be higher, reaching up to 80% or even more if agroindustrial by-products are used as substitutes for cereals in concentrate formulas.

In vitro methane evaluation was carry out maintaining the *in vivo* experimental design. Rumen content was individually collected immediately after slaughter, which took place without prior fasting. To ensure that the rumen inocula were adapted to the diets, each diet was incubated with the rumen content of the animals to which it was allocated.

Compared to the MBp diet, CH_4 yields were reduced by 27.3% and 45.9% with MBpSS and HBpSS, respectively. This reduction can be attributed to a direct effect of the lipids on the ruminal ecosystem, as ether extract in MBpSS and HBpSS almost doubled the values of the MCe and MBp diets. The effects of lipid supplementation on CH_4 reduction has been reported in previous studies (Beauchemin et al., 2007; Beck et al., 2019; Hristov et al., 2013; Johnson & Johnson, 1995), being associated with a reduction in voluntary intake (Rabiee et al., 2012) and an inhibitory effect on rumen *archaea*, bacteria and ciliate protozoa (Vargas, 2020). The reduction of CH_4 production observed in the *in vitro* trial when sunflower seed was supplemented resulted from the decrease in total gas production, and this effect was particularly evident in the diet with 75% haylage. Lower gas production suggests a reduction of organic matter (OM) fermented in the rumen, although the absence of effects on VFA production indicates that fermentative activity was not depressed. Maia, Fonseca, Oliveira, Mendonça, and Cabrita (2016) reported a reduction in total gas and CH_4 production when forages were incubated *in vitro* using inoculum from animals fed with diets supplemented with 5% sunflower oil compared to inoculum from animals not supplemented with oil.

Carcass traits were not affected by diet and corresponded to the expected pattern of the beef carcass market in south Portugal. The average value obtained for carcass yield in this experiment (56.7%) were similar to the 54% reported for the Alentejana breed (Santos-Silva et al., 2020) and 59% for Limousine yearling bulls (Horcada et al., 2016; Maggiolino et al., 2019), slaughtered with similar weights. The prevailing conformation grade was R (75%). For fatness, all the carcasses were classified into 2 or 3, which are the most valuable grades in the Portuguese market, given consumer preferences for lean meat.

By visual assessment, there were no cases in which ScF colour could be a factor for depreciation of carcasses. Furthermore, colour coordinates for ScF, particularly yellowness (*b**), were clearly below 14.2, which is the threshold value reported by Dunne, Mara, Monahan, and Moloney (2004) for the acceptable yellowness of fat in the Italian market.

Diets did not change meat colour, which only varied over the 14 days of ageing. The increase of lightness (*L**), redness (*a**), yellowness (*b**) and intensity (*C**) in meat throughout the ageing period is in accordance with other reports on colour stability of vacuum packing beef (Insausti et al., 1999; Oliete et al., 2005). However, the colour changes observed in the present work did not seem to compromise the meat acceptability

Table 7
Effects of the diet and fat depot on fatty acid composition (g/100 g of total fatty acids) of intramuscular (LT) and subcutaneous fat (ScF) of crossbred Charolais x Alentejana young bulls.

	LT				ScF				*P*- value		
	Diet				Diet						
	MCe[1]	MBp[2]	MBpSS[3]	HBpSS[4]	MCe[1]	MBp[2]	MBpSS[3]	HBpSS[4]	Diet	Dep[5]	Diet*Dep
TFA[6] (mg/g tissue)	14.3	15.9	16.8	15.6	710.9	801.1	770.9	748.1	0.469	<0.001	0.478
	27.80	27.80	27.80	27.80	27.80	27.80	27.80	27.80			
12:0	0.058	0.081	0.058	0.072	0.062	0.078	0.064	0.078	0.144	0.429	0.830
	0.0082	0.0082	0.0082	0.0082	0.0082	0.0082	0.0082	0.0082			
14:0	2.45	2.97	2.51	2.75	2.58	4.13	3.36	3.75	0.024	<0.001	0.098
	0.274	0.274	0.274	0.274	0.274	0.274	0.274	0.274			
i-15:0	0.16	0.16	0.15	0.14	0.16	0.21	0.16	0.16	0.252	0.072	0.223
	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015			
a-15:0	0.17	0.19	0.16	0.17	0.17	0.24	0.17	0.19	0.189	0.017	0.144
	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020			
c9–14:1	0.33	0.48	0.33	0.34	0.37	0.76	0.48	0.58	0.094	<0.001	0.090
	0.084	0.084	0.084	0.084	0.084	0.084	0.084	0.084			
15:0	0.47	0.51	0.44	0.54	0.43	0.62	0.48	0.64	0.060	0.048	0.301
	0.051	0.051	0.038	0.051	0.051	0.051	0.038	0.051			
i-16:0	0.25	0.24	0.18	0.17	0.21	0.27	0.18	0.18	0.007	0.878	0.374
	0.022	0.022	0.015	0.022	0.022	0.022	0.015	0.022			
16:0	25.7a	28.0a	23.4a	23.4a	22.3a	32.3b	25.8a	25.8a	<0.001	0.265	0.037
	3.46	0.72	0.72	0.72	3.46	0.72	0.72	0.72			
i-17:0	0.28	0.27	0.22	0.21	0.22	0.28	0.22	0.22	0.014	0.287	0.181
	0.021	0.011	0.021	0.021	0.021	0.011	0.021	0021			
*c*7–16:1	0.17	0.18	0.16	0.16	0.14	0.17	0.16	0.20	0.371	0.885	0.079
	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015			
*c*9–16:1	2.00	2.51	1.74	1.75	1.84	3.10	2.25	2.49	0.019	0.016	0.221
	0.241	0.241	0.241	0.314	0.241	0.241	0.241	0.314			
a-17:0	0.40	0.43	0.30	0.25	0.45	0.60	0.41	0.37	<0.001	<0.001	0.592
	0.052	0.052	0.022	0.022	0.052	0.052	0.022	0.022			
17:0	1.23	1.17	0.94	0.90	1.02	1.22	0.99	0.92	0.006	0.693	0.627
	0.154	0.063	0.063	0.056	0.154	0.063	0.022	0.056			
i-18:0	0.16	0.15	0.09	0.09	0.13	0.16	0.10	0.08	<0.001	0.475	0.158
	0.013	0.013	0.011	0.011	0.013	0.013	0.011	0011			
*c*9–17:1	0.62	0.62	0.44	0.45	0.47	0.61	0.49	0.44	0.031	0.313	0.136
	0.064	0.042	0.042	0.064	0.064	0.042	0.042	0064			
18:0	18.3	16.8	20.2	20.7	14.4	16.6	20.5	20.6	0.024	0.321	0.527
	1.96	1.07	1.07	1.96	1.96	1.07	1.07	1.96			
*c*11–18:1	1.20c	1.06bc	0.93b	0.88b	0.65a	0.74ab	0.75ab	0.69a	0.450	<0.001	0.014
	0.078	0.078	0.078	0.078	0.078	0.078	0.078	0.078			
*c*9–18:1	31.3	31.4	29.9	27.5	24.3	31.1	32.6	29.8	0.157	0.686	0.061
	4.00	1.06	1.06	1.06	4.00	1.06	1.06	1.06			
18:2n-6	5.59	4.57	6.25	6.47	1.16	1.42	1.77	1.83	0.095	<0.001	0.308
	0.469	0.469	0.469	0.469	0.469	0.469	0.469	0.469			
*c*9–19:1	0.12	0.11	0.06	0.07	0.10	0.12	0.08	0.08	<0.001	0.693	0.278
	0.011	0.011	0.011	0.011	0.011	0.011	0.011	0.011			
20:0	0.14	0.15	0.15	0.15	0.12	0.13	0.12	0.13	0.719	<0.001	0.952
	0.011	0.011	0.011	0.011	0.011	0.011	0.011	0.011			
18:3n-3	1.76	1.60	1.29	1.42	0.61	0.86	0.59	0.62	0.069	<0.001	0.161
	0.111	0.111	0.111	0.111	0.111	0.111	0.111	0.111			
20:4n-6	1.38	1.02	1.28	1.34	0.03	0.02	0.02	0.03	0.546	<0.001	0.551
	0.134	0.134	0.134	0.134	0.134	0.134	0.134	0.134			
20:5n-3	0.41	0.32	0.32	0.36	0.03	0.02	0.02	0.03	0.728	<0.001	0.766
	0.044	0.044	0.044	0.044	0.044	0.044	0.044	0.044			
22:5n-3	0.71	0.59	0.53	0.60	0.02	0.02	0.02	0.03	0.461	<0.001	0.523
	0.059	0.059	0.059	0.059	0.059	0.059	0.059	0.059			
Even-chain SFA	46.6	48.0	46.4	47.1	39.5	53.3	49.9	50.4	0.096	0.584	0.351
	6.14	0.97	0.97	0.97	6.14	0.97	0.97	0.97			
Odd-FA	3.40	3.43	2.70	2.71	2.98	3.84	2.96	2.98	0.026	0.391	0.457
	0.315	0.315	0.136	0.136	0.315	0.315	0.136	0.136			
BCFA [7]	1.41	1.44	1.10	1.04	1.34	1.76	1.23	1.20	<0.001	0.059	0.080
	0.179	0.075	0.075	0.075	0.179	0.075	0.075	0.075			
PUFAn-6 [8]	7.36	5.91	7.92	8.20	1.24	1.49	1.83	1.90	0.184	<0.001	0.369
	0.626	0.626	0.626	0.626	0.626	0.626	0.626	0.626			
PUFAn-3[9]	3.06	2.65	2.26	2.50	0.66	0.93	0.65	0.70	0. 272	<0.001	0.248
	0.214	0.214	0.214	0.214	0.214	0.214	0.214	0.214			

[1] 60% DM of haylage and 40 % DM of concentrate with cereals.
[2] 60% DM of haylage and 40 % DM of concentrate with cereals + by-products.
[3] 54% DM of haylage, 36 % DM of concentrate with cereals + by-products and 10% DM of sunflower seed.
[4] 67.5% DM of haylage, 22.5 % DM of concentrate with cereals + by-products and 10% DM of sunflower seed.
[5] Fat Depot
[6] Total fatty acids
[7] Branched chain fatty acids = iC150+aC150+iC160+iC170+aC170+iC180.
[8] PUFAn-6 = C18:2n6+C202n6+C203n6+C204n6).

[9] PUFAn-6 = C183n3+C205n3+C225n3; values with different superscripts are significantly different ($P<0.05$).

Table 8
Effects of the diet and of fat depot on biohydrogenation intermediate isomers proportion (g/100 g of total fatty acids) of intramuscular (LT) and subcutaneous fat (ScF) of crossbred Charolais x Alentejana young bulls.

	LT				ScF				*P*- value		
	Diet				Diet				Diet	Dep	Diet*Dep
	MCe[1]	MBp[2]	MBpSS[3]	HBpSS[4]	MCe[1]	MBp[2]	MBpSS[3]	HBpSS[4]			
t6-,t7-,t8–18:1	0.17a	0.15a	0.40b	0.47b	0.16a	0.17a	0.50bc	0.55c	<0.001	0.004	0.038
	0.030	0.030	0.030	0.030	0.030	0.030	0.030	0.030			
t9–18:1	0.18	0.16	0.37	0.41	0.16	0.17	0.41	0.46	<0.001	0.029	0.075
	0.021	0.018	0.021	0.021	0.021	0.018	0.021	0.021			
t10–18:1	0.48	0.34	0.70	0.79	0.58	0.54	1.10	1.07	0.005	<0.008	0.282
	0.116	0.127	0.127	0.116	0.116	0.127	0.127	0.116			
t11–18:1	0.73a	0.81a	1.83b	2.62c	0.60a	0.97a	2.15bc	3.30d	<0.001	0.001	0.002
	0.187	0.187	0.213	0.213	0.187	0.187	0.213	0.213			
c12–18:1	0.23	0.20	0.68	0.77	0.19	0.21	0.68	0.78	<0.001	0.844	0.639
	0.048	0.048	0.048	0.048	0.048	0.048	0.048	0.048			
t12–18:1	0.30	0.27	0.61	0.71	0.18	0.19	0.56	0.71	<0.001	0.007	0.266
	0.053	0.046	0.053	0.053	0.053	0.046	0.053	0.053			
c13–18:1	0.13	0.16	0.15	0.10	0.12	0.16	0.12	0.11	0.297	0.634	0.656
	0.022	0.022	0.022	0.022	0.022	0.022	0.022	0.022			
c14-,t16–18:1	0.26	0.28	0.47	0.47	0.25	0.31	0.56	0.61	<0.001	0.001	0.882
	0.044	0.035	0.035	0.044	0.044	0.035	0.035	0.044			
c15–18:1	0.10	0.10	0.16	0.13	0.12	0.14	0.17	0.19	0.004	0.002	0.334
	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.014			
c16–18:1	0.07	0.06	0.11	0.10	0.05	0.04	0.09	0.11	<0.001	0.022	0.246
	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010			
c9, t15–18:2	0.12a	0.12a	0.18b	0.14ab	0.09a	0.11a	0.19b	0.20b	0.001	0.442	0.012
	0.017	0.017	0.016	0.016	0.017	0.017	0.016	0.016			
t11, c15–18:2	0.11a	0.14b	0.09a	0.10a	0.10a	0.14b	0.10a	0.13b	0.012	0.182	0.038
	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010			
c9,t11–18:2	0.20a	0.25ab	0.44c	0.55d	0.20a	0.30b	0.53d	0.73e	<0.001	<0.001	0.002
	0.036	0.036	0.040	0.040	0.036	0.036	0.040	0.040			
TBI 18:1	2.85	2.70	5.84	6.98	2.56	3.08	6.67	8.31	<0.001	0.055	0.507
	0.440	0.366	0.366	0.440	0.440	0.366	0.366	0.440			
TBI 18:2	0.75a	0.79a	1.08b	1.17b	0.63a	0.80a	1.24c	1.48d	<0.001	0.012	0.002
	0.071	0.064	0.071	0.071	0.071	0.064	0.071	0.071			
Delta 9	0.34	0.35	0.31	0.33	0.36	0.33	0.33	0.32	0.767	0.777	0.524
	0.022	0.022	0.022	0.022	0.022	0.022	0.022	0.022			
t10−/t11–18:1	0.69	0.42	0.47	0.31	0.97	0.58	0.71	0.36	0.062	0.005	0.194
	0.146	0.146	0.146	0.115	0.146	0.146	0.146	0.115			

[1] 60% DM of haylage and 40% DM of concentrate with cereals.
[2] 60% DM of haylage and 40% DM of concentrate with cereals + by-products.
[3] 54% DM of haylage, 36% DM of concentrate with cereals + by-products and 10% DM of sunflower seed.
[4] 67.5% DM of haylage, 22.5% DM of concentrate with cereals + by-products and 10% DM of sunflower seed; values with different superscripts are significantly different ($P < 0.05$).

by consumers, since a^* and C^* values exceed threshold values of 12 or 14.5 for redness and 16 for vividness, which consumers considered acceptable for beef (Holman, van de Ven, Mao, Coombs, & Hopkins, 2017; Van Rooyen, Allen, Crawley, & O'Connor, 2017).

Meat lipid oxidation depends on the balance between pro-oxidant and antioxidant compounds, and apparently on the days of cooking, the beef had similar pro-oxidant and antioxidant balance, as there were no effects of diet or ageing time on TBARS. As expected, the lipid oxidation was higher in cooked beef slices after 3 days of storage than immediately after cooking. Despite the increase of lipid oxidation in cooked meat over 3 days of storage, the cooked meat from bulls fed both sunflower seed supplemented diets (MBpSS and HBpSS) were more able to resist of lipid oxidation, showing lower TBARS values than meat from other diets. As intramuscular fat and PUFA levels were similar between diets, the lower lipid meat oxidation in MBpSS and HBpSS can probably be explained by the higher consumption of α-tocopherol and β-carotene observed in these groups.

Intramuscular fat in LT was not affected by diet, averaging of 22.3 ± 4.9 g/kg of meat, and can be classified as lean meat (< 50 g/kg) (Food Advisory Comittee, 1990). These results agree with others reported by Horcada et al. (2016) or Santos-Silva et al. (2020) in young bulls slaughtered with similar weights.

Animals fed diets supplemented with 10% DM of whole sunflower seed (MBpSS and HBpSS), had twice the concentration of fat (8.1 ± 0.38% *vs.* 4.0 ± 0.45%) than those fed the unsupplemented diets (MCe and MBp). In addition to the reduction in CH_4 emissions suggested by *in vitro* data, the increased availability of PUFA in the rumen can modulate ruminal lipid metabolism reflected by the FA composition of tissues. The proportion of odd and branched-chain (OBCFA) in meat were depressed by lipid supplementation. Most OBCFA have a microbial origin (Vlaeminck, Fievez, Cabrita, Fonseca, & Dewhurst, 2006). Therefore, the lower proportion of OBCFA observed in the meat of animals supplemented with sunflower seed may reflect the reduction in intestinal absorption, probably due to changes in the rumen microbiota (Buccioni, Decandia, Minieri, Molle, & Cabiddu, 2012), or greater dilution of OBCFA in the digesta caused by the increased FA intake.

In diets supplemented with whole sunflower seeds, the intake of 18:2n-6 increased 956% for MBpSS and 687% for HBpSS compared to MCe and MBp. Intake of 18:3n-3 also increased but more modestly (26% for MBpSS and 30% for HBpSS). However, effects of diet on 18:2n-6 and 18:3n-3 in LT and ScF did not reach significance, reflecting the extensive BH process in the rumen. Linoleic and linolenic acids are essential FA

and the recommendations of FAO (2010) for human nutrition is that the contribution of n-6 PUFA should vary between 2.5 and 9% of daily energy intake and for n-3 PUFA should vary between 0.5 and 2%. The human diet in developed countries is frequently unbalanced regarding the n-3 PUFA, and the importance of increasing the proportion of these FA in ruminant meat has been highlighted in the literature (Vahmani et al., 2020). Assuming 2511 kcal/day as the energy (E) requirements of a median man and the upper limits referred by FAO (2010) for n-6 PUFA (9% E) and n-3 PUFA (2% E), a serving of 150 g of meat from this experiment would cover about 6.5 ± 0.12% of the daily energy requirements, 7.7 ± 0.68% of total PUFA, 6.1 ± 0.77% of n-6 PUFA and 10.0 ± 0.72% of n-3 PUFA. Diets rich in biodiverse haylage allowed the production of meat whose contribution to meeting the daily human needs of n-3 PUFA was greater than that of n-6 PUFA or total PUFA, resulting in a healthier meat FA profile.

The diets used in this experiment had high fibre and low starch content and in such conditions, *t*11–18:1 would be expected to be the prevalent BI formed in the rumen (Alves et al., 2021; Bessa et al., 2015). The values obtained for *t*10−/*t*11–18:1 ratio confirmed the expected results, as the average values observed for all diets were clearly below 1. Reinforcing the indication given by the average values, only 3 of the 32 animals presented *t*10−/*t*11–18:1 ratios higher than 1 in ScF and LT, confirming that high-forage diets used in this experiment effectively prevented the *t*10-shift (*i.e.* when the t10−/t11–18:1 ratio is >1). Supplementation with whole sunflower seeds proved to be an effective strategy to increase deposition of *t*11–18:1 and *c*9,*t*11–18:2 in beef. Results observed for MBp and MBpS diets show that supplementation with 10% sunflower seed increased the proportions of most BI in the total FA of meat, but mainly *t*11–18:1 and *c*9, *t*11–18:2 which increased 125 and 76%, respectively. The stimulating effect in the deposition of healthy BI when lipid supplements are used in forage based diets was also reported by Angulo et al. (2012), who reported an increase of 216 and 66% in *t*11–18:1 and *c*9, *t*11–18:2, in meat from German Holstein cows.

Most often, animals fed high forage diets present a more complete BH (*i.e.* yielding more 18:0 and less BI) than animal fed diets with high concentrate incorporation (Vasta et al., 2009). In fact, an incomplete BH pattern, yielding high *trans*-18:1 rumen outflows has been related to the presence of environmental stress in the rumen ecosystem associated with low pH or high PUFA (Bessa, Santos-Silva, Ribeiro, & Portugal, 2000). Thus it is surprising that feeding sunflower seed induced the highest *t*11–18:1 and 18:1 BI accumulation in the tissues when added to the basal diet with the 75% forage incorporation than to that with 60%. It is not clear why this happened but the highest intake of total phenol with HBpSS than with MBpSS diet might have contribute to that. In fact, plant phenolic compounds and tannins in particular have the potential to modulate BH, decreasing its completeness (Alves et al., 2017; Frutos et al., 2020).

5. Conclusions

Biodiverse haylage based diets for the finishing of crossbred Charolais × Alentejana young-bulls allowed productivity indexes that are industry typical. Increasing of the forage: concentrate ratio from 60:40 to 75:25 reduced average daily growth rate but had no impact in feed conversion ratios or feeding costs. The reduction of cereals in the concentrate fraction had no effect on animal productivity or carcass and meat quality. The inclusion of 10% DM as whole sunflower seed in the diets was an effective way of supplementing lipid as it 1) had no effect on feed intake or growth performance; 2) reduced *in vitro* CH_4 production; 3) increased intake of α-tocopherol and β-carotene and the oxidative stability of meat 3 days after cooking; 4) resulted in higher proportions of vaccenic and rumenic acids in intramuscular and subcutaneous fat.

Author statement

All authors participated in writing this paper. All authors read and agreed on the final version of the manuscript.

Declaration

Concerning the manuscript Effects of partial substitution of grain by agroindustrial byproducts and sunflower seeds supplementation in beef haylage-based finisher diets on growth, *in vitro* methane production and carcass and meat quality, submitted to Meat Science for publication, the authors declare that there are no conflict of interests. Financial support was provided through the project PDR2020-101-031179, cofinanced by FEADER, Portugal 2020

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