EFFECTS OF DIETARY INCLUSION OF SUNFLOWER SOAPSTOCKS ON COLOR AND MICROBIOLOGICAL FOOD-SAFETY OF MEAT FROM LIGHT FATTENING LAMBS

Sonia Andrés[1], Raúl Bodas[2], José M. Rodríguez-Calleja[3], Carolina Blanco[1], Rodrigo Peláez[1], and F. Javier Giráldez[1]

[1] Instituto de Ganadería de Montaña (CSIC-Universidad de León), E-24346 Grulleros (León), Spain

[2] Instituto Tecnológico Agrario de Castilla y León. Finca Zamadueñas. Ctra. Burgos, km 119. E-47071 Valladolid, Spain

[3] Departamento de Higiene y Tecnología de los Alimentos, Universidad de León, E-24071 León, Spain.

Abstract – Forty-four Merino lambs (6 to 8 wk old; BW 15.6 ± 0.21 kg) were used to study the effect of adding different proportions of sunflower soapstock (SS) to pelleted total mixed ration (TMR) for fattening lambs on color and presence of enteropathogenic bacteria. Lambs were assigned to one of four experimental groups (11 lambs per group), each randomly assigned to one dietary treatment: 00SS (0 g SS/kg TMR pellet), 15SS (15 g SS/kg TMR pellet), 30SS (30 g SS/kg TMR pellet) and 60SS (60 g SS/kg TMR pellet). Lambs were fed individually the corresponding diet *ad libitum*. When lambs reached 27 kg BW they were slaughtered. The longissimus lumborum (LL) samples of 60SS and/or 30SS animals revealed higher discoloration (hue angle) and proliferation of diarrheagenic *E. coli* when compared to the 00SS and 15SS groups. Both results jointly suggest that sunflower soapstoks should not be included in TMR of light fattening lambs at rates higher than 1.5%.

Key Words – dietary by-products, shelf-life, diarrheagenic *E. coli*

I. INTRODUCTION

"Resource efficiency" has been highlighted in Horizon 2020 as one of the key areas for research and innovation, being one of its goals the improvement of the efficiency of feed chains. This strategy embraces the utilization of by-products of the food industry since these alternative feed resources do not compete with food for humans, reduce feeding costs and cope with the need for recycling waste material [1].

In this sense, soapstocks (SS) are a by-product from the vegetable oil refining industry [2], and have been proposed as an economical lipid supplement for ruminants [3] which also help to reduce ruminal methane production [4]. However, the effect of the inclusion of this by-product on meat quality parameters should be properly characterized before recommending the inclusion of SS in compound feeds or total mixed rations (TMR) for ruminants. This is especially important since the fatty acid (FA) composition of meat from lambs being fed SS is modified [5] and, consequently, shelf-life extension (color and lipid oxidation) of meat might be reduced [6]. Additionally, the proliferation of some bacteria might be affected by an increase in unsaturated fatty acids [7]. Therefore, the aim of the present study was to investigate the color and **diarrheagenic *E. coli*** on meat samples during refrigerate storage under modified-atmosphere packaged (MAP) when different doses of SS were included in the diet of light fattening lambs.

II. MATERIALS AND METHODS

After stratification on the basis of body weight (average BW = 15.6 ± 0.21 kg), 44 Merino lambs were penned individually (2.2 m^2 per lamb) and allocated randomly to one of four different groups according to the amount of SS offered in the diet: 00SS (0 g SS/kg TMR pellets), 15SS (15 g SS/kg TMR pellet), 30SS (30 g SS/kg TMR pellets) and 60SS (60 g SS /kg TMR pellet). Each lamb was fed the corresponding experimental diet *ad libitum* (TMR pellets) and fresh drinking water was always available. Ingredients and chemical composition of the feeds are shown in Table 1. The amount of feed offered was adjusted daily on the basis of previous day's intake, allowing refusals of ca. 200 g/kg offered. All handling practices followed the recommendations of European Council Directive 86/609/EEC (European Commission, 2010) for the protection

of animals used for experimental and other scientific purposes, and all of the animals were able to see and hear other lambs. When animals reached 27 kg of BW they were slaughtered by stunning and exsanguination from the jugular vein, prior to being eviscerated and skinned. After 24 h at 4 ºC, the *longissimus lumborum* (LL), was removed from both sides of the carcass to carry out the analyses as described below.

Table 1. Ingredients and chemical composition of experimental total mixed rations.

	Experimental diets[2]			
	00SS	**15SS**	**30SS**	**60SS**
Ingredients (g/kg)				
Barley	433	417	404	375
Corn	150	145	140	130
Soybean meal 44	237	243	246	255
Barley straw	150	150	150	150
SS[1]	0	15	30	60
Mineral/vitamin	30	30	30	30
Chemical composition (g/kg DM)				
Dry matter (g/kg)	900	896	897	897
NDF[3]	227	219	218	212
ADF[4]	121	117	117	110
Crude Protein	174	178	178	182
Fat	30.1	40.7	56.0	69.9
Ash	68	69	67	72

[1]SS= Sunflower soapstock. [2]00SS= TMR pellet without SS. 15SS=TMR pellet containing 15 g SS/kg. 30SS= TMR pellet containing 30 g SS/kg. 60SS=TMR pellet containing 60 g SS/kg. [3]Neutral detergent fiber. [4]Acid detergent fiber.

Color evolution

The LL from the right and left carcass sides, were cut into 2.5 cm thick slices, placed on impermeable polypropylene trays (6 trays with 2 slices per treatment), wrapped with ML40-G bags (Krehalon; Proveedora Hispano Holandesa S.A. Barcelona, Spain) which were immediately modified-atmosphere packaged (MAP) using a tabletop Multivac A300 packaging machine (Multivac Verpackungsmaschinen, Wolfertschwenden, Germany). The air in the bags was replaced by a commercial gas, blend intended for red and poultry meat consisting of 35% CO_2; 35% O_2 and 30% N_2. The ML40-G bags had O_2 and CO_2 transmission rates of 20 and 100 mL m^{-2} 25 h^{-1}, respectively, at 23 ºC and 80% relative humidity. All packages were stored under simulated retail display conditions (12 h daily fluorescent illumination (34 W) and 3 ± 1 ºC) to study the rate of discoloration during 14 days of refrigerated storage.

Two slices of LL for each animal was unpackaged and measured for color parameters on days 0, 1, 3, 7, 9 and 14. The L* (lightness), a* (redness) and b*(yellowness) values (Centre Internationale de l'Eclairage, 1986) were used to determinate the meat color of the muscles using a chromameter (Minolta ® Chroma Meter 2002, Germany). The aperture diameter was 8 mm and illuminant D65 and 10º standard observer were used. The colorimeter was previously calibrated with a pure white colour tile. The hue angle (h*), which defines colour (0º is red; 90º is yellow), was calculated as arctangent (b*/a*), and the chroma (C*), a measure of color intensity (0 is dull; 60 is vivid), was computer as √((a*2+b*2)) (Young and West, 2001).

Detection of virulence genes

a) Target virulence genes

The *stx1*, *stx2* and *eae* genes were selected to estimate the occurrence of diarrheagenic *Escherichia coli* included in the Shiga-toxin-producing *E. coli* (STEC) or enteropathogenic *E. coli* (EPEC) pathogenic groups. STEC strains can cause sporadic cases and disease outbreak in humans, and harbor *stxs* genes encoding the shiga-like toxins (*Stxs*). EPEC strains are an important group characterized by the presence of the locus of enterocyte effacement (LEE) which contains the *eae* gene but do not produce *Stxs*. This important virulence determinant, the LEE region, is also shared by some STEC strains (*eae+*).

b) Extraction of genomic DNA

Genomic DNA was extracted from both lamb meat enrichments and isolates according to adapted method previously described [8], taking into account ISO 22174 general requirements and ISO 22838 [9, 10]. Briefly, enrichments were obtained from homogenates of 10 g lamb meat in 90 ml trypticase soy broth (TSB) plus 0.6% yeast extract (Oxoid), after overnight incubation at 30 ºC and with constant shaking (150 rpm). An aliquot of the enriched sample was streaked onto SMAC (Oxoid) plates and incubated at 37°C for 24h. Both pink (sorbitol fermenters) and colorless (non-fermenters) colonies were picked and cultivated in TSB.

c) PCR assay

An aliquot of 1 ml was taken from meat enrichments and isolates respectively, and centrifuged at 9000g for 10 min. The pellet was washed three times with phosphate-buffered saline (Oxoid) and resuspended in 500 ml of a mixture of distilled water and 1% Triton X-100 (Sigma, Barcelona Spain), and DNA was extracted by a boiling procedure and purified with the Illustra bacteria genomicPrep Mini Spin Kit (GE Healthcare Life; Buckinghamshire, UK).
The template DNA was screened by PCR for the presence of the target genes using the primers described in Paton &Paton [11]. PCR was carried out in a Mastercycler Personal (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) in a final volume of 50 µl and an annealing temperature of 61 °C (*stx1* and *stx2* genes) and 59 ºC (*eae* gene). PCR products were analyzed by 1% agarose gel electrophoresis and viewed after ethidium bromide staining under UV light.

III. RESULTS AND DISCUSSION

Table 2 summarizes the results of LL muscle when the color stability was studied after packaging at refrigerated storage for 14 days.

Table 2. Evolution and mean values of color parameters of meat samples (4 ºC, 14 days).

		L*	A*	b*	C*	h*
Group[1]	00SS	45.2^{a}	10.3^{ab}	5.7^{a}	11.7	28.9^{ab}
	15SS	44.2^{b}	10.5^{a}	5.3^{b}	11.8	26.8^{c}
	30SS	44.3^{b}	10.5^{a}	5.5^{ab}	11.9	27.7^{bc}
	60SS	45.4^{a}	9.9^{b}	5.7^{a}	11.5	29.8^{a}
Day	0	45.6^{ab}	9.3^{c}	3.8^{d}	10.1^{d}	22.5^{c}
	1	45.2^{a}	10.0^{b}	6.1^{a}	11.7^{c}	31.3^{a}
	3	44.5^{bc}	11.0^{a}	6.0^{ab}	12.5^{a}	28.7^{b}
	7	43.6^{c}	10.9^{a}	5.5^{c}	12.2^{abc}	26.9^{b}
	9	43.9^{c}	11.1^{a}	5.6^{bc}	12.4^{ab}	27.0^{b}
	14	45.2^{ab}	10.0^{b}	6.1^{a}	11.7^{bc}	31.3^{a}
rsd		1.97	1.03	0.63	0.98	3.54
P-group		0.004	0.011	0.030	0.109	<0.001
P-day		0.002	<0.001	<0.001	<0.001	<0.001
P-group*day		0.459	0.393	0.511	0.352	0.603

[a,b,c] Different superscripts indicate statistical differences between means (P <0.05).

As can be observed, b* (yellowness index) was lower (P = 0.030) in the LL muscle of the lambs fed 15SS when compared to the rest of groups, whereas a* (redness index) was significantly reduced in the 60SS group (P=0.011). Changes in both parameters promoted differences in the hue angle (h*, color) which shifted from yellow to red in the 15SS group, and from red to yellow in the 60SS group (P < 0.001). As far as C* is concerned (color intensity), this parameter was not modified significantly by the diet (P=0.109) whereas L* (lightness) showed significant quadratic differences among groups (P<0.001).

Changes in color might be indicating that meat samples become dull faster in the lambs being fed the highest dose of SS (60SS group). The higher susceptibility of PUFAs to lipid oxidation might have promoted the discoloration of LL samples from 60SS lambs due to the oxidation of ferrous heme–iron (Fe2+) into its ferric form (Fe3+). The transformation of oxymyoglobin into metmyoglobin promotes the shift from a pleasant bright red to an undesirable brownish color. On the contrary, 15SS group showed significant lower values of hue when compared to the control lambs (00SS), so in this case antioxidants (vitamin E or other antioxidants included in the SS) might have neutralized the oxidation promoted by PUFAs.

The microbial analysis (Table 3) indicate high levels of positive samples for the investigated virulence genes by PCR in meat samples being fed the highest doses of soapstocks (30SS and 60SS) when compared to those containing low level of SS (15SS) or control (00SS). These observations were not correlated with *Enterobacteriaceae* counts, being even lower those determined from 60SS meat samples.

Table 3. Effects of SS on PCR amplification of *E. coli* virulence genes in lamb meat samples stored at 4 ºC.

	Positive samples for PCR[1]	Samples with positive isolated strains[2]	EC (log ufc/g)[3]
00SS	1/5	0/1	1.65±0.28
15SS	1/6	0/1	1.78±0.33
30SS	6/6	3/6	1.49±0.28
60SS	4/6	4/4	1.37±0.40

[1]Number of sample enrichments showing PCR amplification of *stx*1 and/or *stx*2 and/or *eae* genes (no. positive samples/no. investigated samples); [2]Number of samples where a strain harbouring *stx*1 and/or *stx*2 and/or *eae* genes was isolated from sample enrichments and using the spread plate technique (no. samples with positive isolated strains/no. positive samples); [3]Average *Enterobacteriaceae* counts in all analyzed samples (at least six samples) throughout refrigerated storage.

These results were confirmed lately when strains harboring *stx1* and/or *stx2* and/or *eae* genes were isolated from sample enrichments using the spread plate technique. Other authors have demonstrated that eicosapentaenoic acid (EPA, a n−3 fatty acid) exerts significant bactericidal and bacteriostatic effects against foodborne and food spoilage microorganisms [7]. However, the results observed in the present study indicate that n−3 fatty acids (or other components included in soapstocks) might have enhanced the proliferation of some enteropathogenic groups. Also, our data reveal that lamb meat from animals fed 15 g/kg soapstocks supplements appears not to raise the diarrheagenic *E. coli* (STEC and EPEC) risk compared to control samples. According to the present study, the appropriate assessment of animal experimental diets should consider not only chemical and physical parameters but also implications for food safety by evaluating food-borne bacteria.

CONCLUSION

The inclusion of sunflower soapstocks in the diet for light fattening lambs up to 15 g/kg does not affect negatively meat color or food safety whereas highest doses seem to promote discoloration and proliferation of **diarrheagenic *E. coli***. The results suggest that the optimum inclusion level of sunflower soapstock in the diet for light fattening lambs would be around 15 g/kg diet.

ACKNOWLEDGEMENTS

Financial support was received from the Spanish Ministry of Science and Innovation, Projects AGL2010-19094 and AGL2011-26118/ALI. Raúl Bodas was supported by a JAE-Doc contract and subprogramme 'Doc-INIA'. Carolina Blanco was supported by a contract for young researchers (Junta de Castilla y León and European Social Fund). The authors declare no conflict of interest.

REFERENCES

1. Vasta, V., Nudda, A., Cannas, A., Lanza, M. & Priolo, A. (2008). Alternative feed resources and their effects on the quality of meat and milk from small ruminant. Animal Feed Science and Technology 147: 223-246.
2. Bender, D. A. (2009). A dictionary of food and nutrition: Oxford University Press.
3. Abel-Caines, S., Grant, R. & Morrison, M. (1998). Effect of soybean hulls, soy lecithin, and soapstock mixtures on ruminal fermentation and milk composition in dairy cows. Journal of Dairy Science 8: 462-470.
4. Machmüller, A., Ossowski, D. A., Wanner, M. & Kreuzer. M. (1998). Potential of various fatty feeds to reduce methane release from rumen fermentation in vitro (Rusitec). Animal Feed Science and Technology 71: 117-130.
5. Blanco, C., Giráldez, F. J., Prieto, N., Morán, L., Andrés, S., Benavides, J., Tejido, M. L., & Bodas, R. (2014). Effects of dietary inclusion of sunflower soapstocks on nutrient digestibility, growth performance and ruminal and blood metabolites of light fattening lambs. Journal of Animal Science 92: 4086-4094.
6. Li, Y. & Liu S. (2012). Reducing lipid peroxidation for improving colour stability of beef and lamb: on-farm considerations. Journal of the Science of Food and Agriculture 92: 719-729.
7. Shin, S. Y., Bajpai, V. K., Kim, H. R., Kang, S. C. (2007). Antibacterial activity of eicosapentaenoic acid (EPA) against foodborne and food spoilage microorganisms. LWT-Food Science and Technology 40: 1515–1519.
8. Rodríguez-Calleja, J. M., García-López, M. L., Garcia-Lopez, Santos, J. A. & and Otero, A. (2006). Rabbit meat as a source of bacterial foodborne pathogens. Journal of Food Protection, 69:, 1106-1112.
9. International Organization for Standardization (2006). ISO 20838:2006. Microbiology of food and animal feeding stuffs. Polymerase chain reaction (PCR) for the detection of food-borne pathogens. Requirements for Amplification and Detection for Qualitative Methods. International Organization for Standardization, Geneva.
10. International Organization for Standardization (2005). ISO 22174:2005. Microbiology of food and animal feeding stuffs. Polymerase chain reaction (PCR) for the detection of food-borne pathogens. General requirements and definitions. ISO, Geneva.
11. Paton, A. W. & Paton. J. C. (1998). Detection and characterization of Shiga toxigenic Escherichia coli by using multiplex PCR assays for *stx1*, *stx2*, *eaeA*, enterohemorrhagic *E. coli hlyA*, rfbO111, and rfbO157. Journal of Clinical Microbiology 36: 598–602.