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Effect of muscle type and CO-pretreatment combinations on the colour stability, protein oxidation and shelf-life of vacuum packaged beef steaks

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ABSTRACT

This study investigated the effects of CO-pretreatments on three bovine muscles: *Psoas major* (PM), *Gluteus medius* (GM) and *Semitendinosus* (ST) on colour stability and quality attributes under anaerobic conditions. Steaks were exposed to one of four pretreatments: 1% CO for 5 h, 5% CO for 5 h, 1% CO for 24 h, with 60% CO_2, (balance N_2) or untreated (control) and then vacuum packaged and displayed (28 days at 2 °C). CO pretreatments improved redness for colour labile muscles (PM and GM). The optimum CO pretreatment was 1% for 5 h as this induced surface redness while discolouring ($a^* = 12$, $C^* = 16$) by the use-by-date (28 days). CO pretreatments decreased protein oxidation ($P < 0.01$), and had no negative effect on pH and spoilage bacteria ($P > 0.05$). Applying a 1% CO pretreatment for 5 h effectively enhanced the colour of various muscles while not masking spoilage thus addressing consumer concerns.

1. Introduction

Maintaining an attractive bright red colour which is desirable to consumers combined with excellent eating quality remains a key goal for the meat industry. A solution could be to apply carbon monoxide (CO) as a pretreatment to enhance meat colour coupled with vacuum packaging which is known to improve tenderness and extend shelf-life. CO-pretreatment of beef *Longissimus thoracis et lumborum* (LTL) steaks prior to vacuum packaging enhanced colour stability while allowing discolouration to occur by the use-by date (28 days) so as to not mask spoilage (Van Rooyen, Allen, Crawley, & O'Connor, 2017). The regulation of the use of CO in meat packaging differs globally (Van Rooyen, Allen, Crawley, & O'Connor, 2017). However CO is not regulated within the E.U. due to the possibility of misleading consumers due to the bright red colour being retained beyond the safe consumption date (European Commission, 2001; Cornforth & Hunt, 2008).

It is important that this packaging system be evaluated with different muscles prior to commercialisation. The effects of CO packaging are muscle dependent (Claus, 2005; Mancini, Suman, Konda, & Ramanathan, 2009). Claus (2005) also reported that the use of CO in meat packaging systems may not be effective for all muscles types. Variations in colour stability occur among muscles and within muscle cross-sectional areas (Hunt & Hedrick, 1977). Different muscle groups can vary in colour stability due to differing biochemical (intrinsic) and physical (extrinsic) characteristics (Bekhit & Faustman, 2005; Claus, 2005). Biochemical factors which affect meat colour include mitochondrial density, nicotinamide adenine dinucleotide (NAD) and enzyme activity (Claus, 2005). Each muscle has a unique metabolic role and can vary in metmyoglobin reducing activity (MRA) or oxygen consumption rate (OCR) which influence colour stability (Seyfert, Mancini, Hunt, & Faustman, 2007). High OCR is related to more rapid meat colour discoloration due to increased residual mitochondrial respiration (Bendall & Taylor, 1972). Packaging type influences variability in colour stability for different muscles (Behrends, Mikel, Armstrong, & Newman, 2003b; Mancini et al., 2009).

Rozbeh, Field, and Johnson (1993) applied either 10% CO pretreatments (with the balance being N_2) for 1 h or 100% CO-pretreatment for 30 min to *Semitendinosus* (ST) steaks prior to vacuum packaging and reported increased colour stability. Similarly Brewer, Wu, Field, and Ray (1994) applied 100 % CO-pretreatment to "top round" and ST muscles prior to vacuum packaging and showed improved colour stability. Previous studies by this group have already demonstrated the positive effects of CO-pretreatment prior to vacuum packaging on colour stabile muscles such as the *Longissimus thoracis et lumborum* (LTL) but more research is required on less colour stable muscles. There is limited information exists on the effect of CO-pretreatment on muscles varying in colour stability.

As meat matures, myofibrillar proteins are susceptible to oxidation

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over extended storage resulting in the formation of reactive oxygen species (ROS) (Lund, Heinonen, Baron, & Estevez, 2011). Protein oxidation is associated with physical and chemical activity which contributes to damaged amino acids and reduced protein solubility caused by protein polymerisation, decreased enzyme activity and the production of amino acid derivatives such as carbonyls (Lund et al., 2011). Protein oxidation has been reported to concur with meat discolouration (Utrera, Morcuende, & Estévez, 2014). Other negative effects of protein oxidation include increased meat toughness and degradation of nutritional properties (Zakrys, O'Sullivan, Allen, & Kerry, 2009). Decreased meat tenderness due to protein oxidation has been linked to reduced enzymatic calpain activity required for proteolysis and tenderisation (Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004). Decreased nutritional value of meat via protein oxidation is related to loss of essential amino acids and reduced digestibility (Lund et al., 2011; Soladoye, Juarez, Aalhus, Shand, & Estevez, 2015). Intake of oxidised proteins is also a potential health risk (Estevez & Luna, 2017). There are no studies that report the effect of CO pretreatments on protein oxidation. CO is considered an antioxidant therefore the hypothesis of this paper that CO will reduce protein oxidation.

In this present study, the objective was to determine the effectiveness of different CO-pretreatment combinations on three different bovine muscles: *Psoas major* (PM), *Gluteus medius* (GM) and *Semitendinosus* (ST) by determining their effect on colour stability, pH, CO penetration depth, protein oxidation and spoilage bacteria.

2. Materials & methods

2.1. Sample handling and pretreatment processing

Sample preparation was performed as described previously (Van Rooyen, Allen, Crawley, & O'Connor, 2017) with minor modifications. The *Psoas major* (PM), of pH 5.53-5.68, weight 3.30-3.60 kg, *Gluteus medius* (GM) of pH 5.51-5.66, weight 3.10-3.45 kg and *Semitendinosus* (ST) of pH 5.48-5.58, weight 3.00-3.55 kg (total muscles n = 6). These muscles were extracted from the left and right of the same Charolais-cross (CHX) heifer, between 21-29 months, from a commercial abattoir. After 6-8 days post-mortem ageing, uniform steaks were sliced (2.54 cm thick) and randomised between muscle groups to account for any potential systematic left and right side differences. One steak from each muscle was then placed in each CO-pretreatment pouch (5-layer coextruded film with PA/Tie/PE/Tie/PE (Oxygen Transfer Rate: < -70 cm^3 O_2/m^2/24 h at 23 °C and 50% Relative Humidity, Versatile Packaging, Ltd., Castleblayney, Co. Monaghan, Ireland). These were previously prepared with an inlet and an outlet tube (Z280372, PVC tubing, flexible, ¼ in. × 3/8 in., Sigma- Aldrich, Ireland), connectors (Z17847012EA, Tubing connectors, Sigma- Aldrich, Ireland) and disconnectors (Z1043512EA, Tubing quick disconnectors, Sigma- Aldrich, Ireland) before being vacuum packaged (New Diamond Vac J-V006W, Heavy Duty Automatic Vacuum Machine, Jaw Feng Machinery Co., LTD, Taiwan; vacuum pressure < 0.01 Torr held for 32 s) to allow reduction and the formation of deoxymyoglobin. Steaks in the reduced pouches were assigned to one of four treatments selected from a preliminary trial (Van Rooyen, Allen, Reid, & O'Connor, 2017). The treatments were 1% CO, 60% CO_2 and 39% N for 5 h exposure (1% CO 5 h); 5% CO, 60% CO_2 and 35% N for 5 h exposure (5% CO 5 h); 1% CO, 60% CO_2 and 39% N for 24 h exposure (1% CO 24 h); or the control (untreated vacuum packaged). Treatment combinations investigated for respective muscle and CO-pretreatment combination are presented in Table 1. Pouches were connected to the calibration-grade CO gas mixtures (Air Products and Chemicals, Inc.,) and flushed for a standardised time of 2 min. Pouches were then transferred to a chill room (2° C) for the allocated exposure times when they were removed from the pretreatment pouches and rapidly individually repackaged in BB3055 vacuum packages (Oxygen transfer rate (OTR): < 17 cm^3/m^2 O_2, 24 h at 23 °C, 0% relative humidity, CryoVac, Sealed Air Ltd., Dublin, Ireland). All packs were then randomly placed under simulated retail display at 2° C (Continuous fluorescent lighting, Meat - Fluorescent Touchcoat T5 F18W T8 176 Foodstar Meat Toughcoat, Havells Sylvania Fixtures UK, Ltd, 2115 lux. The temperature was recorded at the surface of the meat packages on each shelf every five minutes using dataloggers (Lascar EasyLog-USB, Lascar Electronics Ltd, Salisbury, SP5, UK). The average temperature ranged from 1.9 to 2.6 °C). This whole experiment was repeated on three separate occasions using three different animals.

Table 1
Effect of muscle type, gas combinations and display days on Hue values of PM, GM, ST steaks.

Display days	Hue	S.E.M.		*P* value
0	33.05[a]	0.578	Muscle	0.001
7	35.82[b]		GasC_Exp	0.001
14	37.59[c]		Day	0.001
21	37.60[c]			
28	37.12[bc]			
GasC_Exp				
Control	38.72[b]			
1% CO 5 h	37.39[b]	0.517		
5% CO 5 h	34.38[a]			
1% CO 24 h	34.45[a]			
Muscle				
PM	34.36[a]	0.448		
GM	34.80[a]			
ST	39.56[b]			

Least square means without a common letter are different (P < 0.05).
Pooled standard error of means (S.E.M).
Psoas Major (PM).
Gluteus medius (GM).
Semitendinosus (ST).
GasC_Exp: CO gas concentration and exposure time combination.
All interactions were not-significant (P > 0.05).

2.2. Instrumental colour analysis

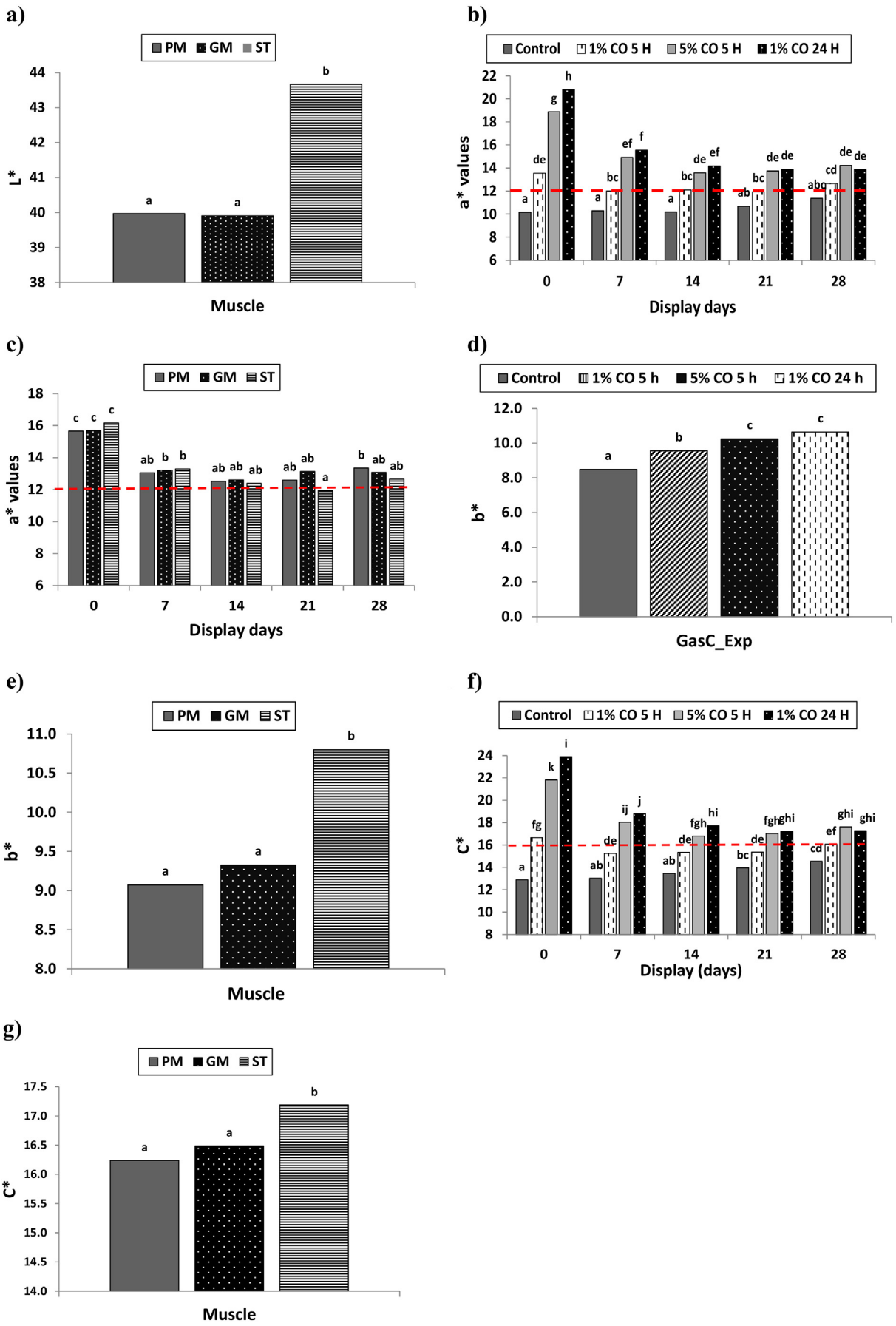
Instrumental surface colour analysis was performed using a HunterLab UltraScan Pro spectrophotometer (Hunter Associates Laboratory., Inc., Reston, VA) standardized using a light trap and white tile that was covered with the same vacuum film to eliminate any packaging effect (AMSA, 2012). Using the 25 mm diameter aperture and illuminant D_{65} with 10° observer, triplicate measurements of CIE L* (lightness), a* (redness) b* (yellowness) were recorded on each steak within the vacuum packages in different separate locations avoiding intramuscular fat and connective tissue within the vacuum packages and averaged. Hue ($\tan^{-1}(b^*/a^*)$) and Chroma ($C^* = (a^{*2} + b^{*2})^{1/2}$) values were calculated (Hunter & Harold, 1987). Colour measurements were recorded at 0, 7, 14 and 28 days display.

2.3. pH

Sample pH was determined using a glass probe pH electrode (Thermo Scientific pH meter 420A, Orion Research Inc.) as described in Van Rooyen, Allen, Gallagher, and O'Connor. Measurements were recorded immediately after steaks were removed from vacuum packages. The pH of each treated steak was recorded at 0, 7, 14, 21 and 28 days display.

2.4. Carboxymyoglobin (COMb) layer depth

Carboxymyoglobin (COMb) is formed when meat is exposed to CO. The depth of the COMb layer was measured according to the method by Raines and Hunt (2010). Upon removal from vacuum packages, samples were sliced laterally and the internal cross-section of the



(caption on next page)

Fig. 1. (a) Effect of muscle type on L* values. PM = *Psoas major*, GM = *Gluteus medius* and ST = *Semitendinosus*. Least square means without a common letter are different ($P < 0.05$). Pooled standard error of means (S.E.M) = 0.38 (b) Effect of CO pretreatment and display day on a* values (averaged for each muscle) steaks stored at 2 °C. Least square means without a common letter are different ($P < 0.05$). Pooled standard error of means (S.E.M) = 0.28 (c) Effect of muscle and display day on a* values. PM = *Psoas major*, GM = *Gluteus medius* and ST = *Semitendinosus* steaks stored at 2 °C. Least square means without a common letter are different ($P < 0.05$). Pooled standard error of means (S.E.M) = 0.24 (d) Effect of CO pretreatment on b* values (averaged for each muscle) steaks stored at 2 °C. GasC_Exp: CO gas concentration and exposure time combination. Least square means without a common letter are different ($P < 0.05$). Pooled standard error of means (S.E.M) = 0.17 (e) Effect of muscle on b* values. PM = *Psoas major*, GM = *Gluteus medius* and ST = *Semitendinosus* steaks stored at 2 °C. Least square means without a common letter are different ($P < 0.05$). Pooled standard error of means (S.E.M) = 0.15 (f) Effect of CO pretreatment and display day on C* values of steaks (averaged for each muscle) stored at 2 °C. Least square means without a common letter are different ($P < 0.05$). Pooled standard error of means (S.E.M) = 0.35 (g) Effect of muscle type on C* values. PM = *Psoas major*, GM = *Gluteus medius* and ST = *Semitendinosus* steaks stored.

transitional point of the COMb surface layer to the deoxymyoglobin (DMb) layer was rapidly recorded (to minimise potential blooming) using a digital calliper (Draper Expert, PVC 150 D, Draper Tools Ltd, Hampshire, SO53, UK). Three separate measurements were recorded for each sample and the mean was obtained. Measurements were taken after 0, 7, 14, 21 and 28 days display.

2.5. Protein carbonyl content

Protein oxidation was determined by the dinitrophenylhydrazine (DNPH) method by calculating the total carbonyl content using a Biocell protein carbonyl enzyme Immuno-assay kit (BioCell Corporation Ltd, Auckland, New Zealand). All reagents were provided by the test kit. Firstly, protein extraction was carried out on steaks that had been frozen (-20° C) at the end of the 28 day display period (35 days post-mortem) according to the procedure by Devries et al. (2008). Two independent samples (1 g approx. 0.1 cm in depth) were excised from the surface layer of each pretreated steak and added to a 25 ml protein extraction buffer (70 mM sucrose, 220 mM mannitol, 5 mM Hepes, pH 7.2, 1 mM EGTA, pH 7.2, and 150 mM KCl) before being homengenised for 1 min using Ultra-turrax T25 homogeniser (Janke & Kunkel GmbH, IKA Labortechnik, Staufen, Germany) set at 13500 rpm while kept on ice. Each homogenate was adjusted to a pH of 7.2 and then centrifuged (LYNX 6000, Thermo Scientific) for 10 min at 2506 × G Force at 4 °C. The supernatants were removed and 1 µl of each supernatant was measured using a Nanodrop (ND-1000 Spectrophotometer) at 280 nm to determine protein content. Protein carbonyl content was then carried out according the Biocell protein carbonyl enzyme Immuno-assay kit manufacturer's guidelines and measured using a plate reader (FLUOstar Omega Microplate Reader, BMG Labtech GmbH, Offenburg, Germany). Protein carbonyl content was calculated using a standard curve and results expressed as nmol DNPH/ mg protein.

2.6. Microbiological analysis

Microbial populations were enumerated at the end of the shelf-life (28 days display at 2 °C) similar to the methods described by Van Rooyen, Allen, Crawley, and O'Connor (2017). The total viable counts (TVCs) for anaerobic mesophiles (TVCm), anaerobic psychrotrophiles (TVCp) and lactic acid bacteria were estimated using a 10 g surface sample (approximately 0.2 cm deep) of each beef CO-pretreated steak which was aseptically excised using a scalpel and suspended into 90 ml of Maximum Recovery Diluent (MRD) (Oxoid LTD, England, CM0733) held in a sterile filter stomacher bag. Results were expressed as $\log_{10}$ CFU (colony forming units)/g of pretreated surface layer of beef.

2.7. Statistical analysis

The entire experiment was repeated three times on separate occasions using a different animal for each replicate. Data were analysed using GenStat using two separate forms of analysis (Release 14.1 Copyright 2011, Lawes Agricultural Trust, Rothamsted Experimental Station, Hertfordshire, UK). For colour data, pH, COMb layer a 3 × 4 × 5 split plot factorial design with three muscles (*Psoas major* (PM), *Gluteus medius* (GM) and *Semitendinosus* (ST)), four gas combinations 5% 5 h, 1% 5 h, 1% 24 h and control (untreated) and five storage times (0, 7, 14, 21, 28 d) as fixed effects and the replicate as a random effect. Protein oxidation and microbiological analysis were analysed separately using a 3 × 4 factorial split plot design including: three muscles (*Psoas major* (PM), *Gluteus medius* (GM) and *Semimembranosus* (SM)), four gas combinations 5% 5h, 1% 5 h, 1% 24 h and control (untreated). Post-hoc tests for both forms of analysis were carried out using Fishers protected LSD and differences were considered ($P < 0.05$).

3. Results and discussion

3.1. Instrumental colour analysis

Results for colour analysis are presented in Fig. 1a–g. There was a muscle effect ($P < 0.001$) on L* values (Fig. 1a). ST had higher L* values ($P < 0.001$) compared to PM and GM. This result is in agreement with findings by Seyfert et al. (2007) and Seyfert et al. (2006) who reported higher L* values in ST muscles compared to PM beef muscles. Similarly, Claus and Du (2013) reported that ST also had higher L* values compared to PM muscles stored under anaerobic packaging with nitrite-embedded film. Variability in myoglobin content between muscles influences the degree of lightness in meat (Calnan, Jacob, Pethick, & Gardner, 2016; Miller, 2002). Myoglobin concentration is directly related to muscle fibre type (red or white) which influences colour (Renerre & Labas, 1987). Therefore, the lighter colour of the ST is possibly due to higher content of white fibres (Hunt & Hedrick, 1977; Seyfert et al., 2007) which are more prone to PSE (pale soft and exudative) (Giddings, 1974). In this study meat colour stability was mainly evaluated through a* and Chroma values and used as an indication of discoloration. Redness (a*) values are presented in Fig. 1b-c and represent the surface redness used as an indicator of freshness by consumers. There was a CO pretreatment × display day interaction ($P < 0.001$) with increased a* values being associated with increased CO gas concentration (5% CO) and increased exposure time (24 h) with the magnitude of this effect decreasing over storage (Fig. 1b). The optimum CO-pretreatment may be 1% CO for 5 h exposure as at 28 days the a* value was close to 12 (threshold for discoloration), however this was not significantly different from the other pretreatments. (Fig. 1b). This result supports previous work (Van Rooyen, Allen, Reid, & O'Connor, 2017). This value was selected based on MacDougall, Down, and Taylor (1986) who reported C* = 16 is the limit of acceptability using a Hunterlab (illuminant D) and this value is equivalent to a* = 12 (Van Rooyen, Allen, Crawley, & O'Connor, 2017) (Fig. 1b). This result indicates that CO pretreatment may be effectively applied to other muscles to enhance colour while not masking spoilage.

There was a muscle × display day interaction ($P < 0.01$) with a* values decreasing over display with the ST being less red than the other two muscles at 21 days while the PM was more red at 28 days (Fig. 1c). By the end of shelf-life (day 28) all muscles discoloured. The relative position of colour by use-by date (28 days) ranked in the following order PM > GM > ST. Similar findings were reported by Suman, Mancini, Ramanathan, and Konda (2009) where PM steaks were redder than *Longissimus lumborum* (LL) steaks stored under 0.4% CO-MAP. Likewise, Liu et al. (2014) showed PM muscles in 0.4% CO-MAP had increased redness compared to LL steaks in the same packaging system.

Table 2
Effect of muscle and gas pretreatment combinations for TVC, lactic acid bacteria, on PM, GM and ST beef

	Treatments														
	PM				GM				SM						
Bacteria	Control	1% 5H	5% 5H	1% 24H	Control	1% 5H	5% 5H	1% 24H	Control	1% 5H	5% 5H	1% 24H		*P* value	S.E.M
TVCm	5.38	5.19	4.93	2.93	6.41	2.70	3.07	2.28	4.93	4.40	2.81	4.45	Muscle	0.774	0.97
													GasC	0.499	1.13
													Muscle*GasC	0.907	1.95
TVCp	7.06	7.04	6.63	6.29	6.92	6.82	6.62	6.51	6.47	6.36	6.76	6.38	Muscle	0.280	0.12
													GasC	0.204	0.14
													Muscle*GasC	0.538	0.24
LAB	4.42	4.47	4.47	2.96	6.14	4.35	4.08	3.88	4.41	4.34	4.27	4.03	Muscle	0.785	0.54
													GasC	0.521	0.63
													Muscle*GasC	0.922	1.09

TVCm: Anaerobic mesophile.
TVCp: Anaerobic psychrotrophiles.
LAB: Lactic acid bacteria.
Psoas Major (PM).
Gluteus medius (GM).
Semitendinosus (ST).
Statistical significance: ($P < 0.05$).
GasC: pretreatment.
S.E.M: Standard error of means
There was no significant main effects or interactions observed ($P < 0.05$).

CO-pretreatment had an effect ($P < 0.001$) on yellowness (b*) values which increased with extended exposure time (24 h) (Fig. 1d & f). The CO pretreatment with the highest yellowness values was 1% CO for 24 h, followed by 5% CO 5 h (10.64), 1% CO 5 h (9.55) and the control (8.94) (Fig. 1d). Muscle had an effect ($P < 0.001$) on b* values with ST being the most yellow, GM and PM the least yellow (Fig. 1e). Similar results were evident in a study by (Seyfert et al., 2007) as ST steaks stored in 0.4% CO-MAP were more yellow than PM. There was no storage effect observed for b* value ($P > 0.05$).

Hue values are used as an index of meat browning or discoloration (Van Rooyen, Allen, Crawley, & O'Connor, 2017). Hue values were affected by muscle ($P < 0.001$), CO pretreatment ($P < 0.001$), and day ($P < 0.001$) (Table 2). Hue values generally increased over storage, with higher CO gas concentration (5% CO) or increased exposure time (24 h) having the lowest hue values (Table 1). PM had the lowest hue values, GM intermediate and ST had higher hue values ($P < 0.05$) compared to PM and GM (Table 2). In contrast Isdell, Allen, Doherty, and Butler (1999) reported similar hue values for GM and ST beef steaks in overwrap packages stored in a modified atmosphere packaging (MAP) mother pack systems with oxygen scavengers. However, the results from this study differ as ST was more yellow than GM and PM ($P < 0.05$). This result indicates that the CO pretreatment may improve the colour stability of colour labile muscles such as PM and GM.

Chroma (C*) is used as a measurement of colour intensity and increased C* values represent a more vivid colour. C* values followed a similar pattern to a* values. A CO pretreatment × display day interaction occurred ($P < 0.001$) with increased CO concentration (5% CO) or extended exposure time (24 h) having increased C* values and decreasing over storage (Fig. 1f). The optimum CO-pretreatment was 1% CO for 5 h (16.08) which was consistent with a* values in showing that the initial colour was enhanced while discolouration occurred by the use-by date as C* values fell to just above the limit of acceptability ($C > 16$) (MacDougall et al., 1986) by day 28 (Fig. 1f). There was also a significant muscle effect ($P < 0.001$) evident with ST being the most colour stable, GM intermediate and PM the least stable (Fig. 1g). Similar findings were reported by (Seyfert et al., 2007) as ST steaks were more colour stable (higher C* values) than PM stored under 0.4% CO-MAP. PM is well documented as being considered to be a colour labile muscle due to increased mitochondria and a high oxygen consumption rate (OCR) (Joseph, Suman, Rentfrow, Li, & Beach, 2012; Mancini et al., 2009; O'Keeffe & Hood, 1982). Likewise, GM has also been shown to have poor colour stability, high OCR and high mitochondrial content (Hunt & Hedrick, 1977; Renerre, 1984). In contrast, ST is colour stable (Behrends et al., 2003). Differences in colour stability between muscles are largely dependent on intrinsic biochemical characteristics including MRA and OCR within myoglobin (Mancini et al., 2009; Seyfert et al., 2007). There have been no reports on the influence of CO pretreatments on varying muscle including GM. The results obtained, (PM and GM having slightly improved colour compared to ST in this study) are not what one would expect in colour labile muscles if an oxygen packaging system had been used. It is therefore possible that CO pretreatment of colour labile muscles with CO influences the rate of MRA and OCR and therefore causes a reverse effect. Therefore it appears that PM and GM did not have poorer colour stability as one would expect when exposed to an oxygen environment. Since PM and GM have a high OCR, the lack of oxygen available to scavenge in the anoxic environment (CO pretreated vacuum pack environment) used in this study, may have supported an extended colour life for colour labile muscles. CO-MAP has been reported to reduce OCR through mitochondrial respiration inhibition (Atkinson & Follett, 1973; Seyfert et al., 2007). Therefore it can be concluded from the results in this present study that CO pretreatment can improve colour labile muscles such as PM and GM which characteristically have high OCR. This result supports Mancini et al. (2009) who reported similar findings in CO-MAP for PM muscles.

3.2. pH

Monitoring meat pH is an important determinant of meat quality especially in terms of meat colour (AMSA, 2012). There was no muscle ($P > 0.05$) or CO pretreatment combination ($P > 0.05$) effect on pH in agreement with previous work by this group and others (Aspé, Roeckel, Martí, & Jiménez, 2008; Sakowska, Guzek, & Wierzbicka, 2016; Van Rooyen, Allen, & O'Connor, 2016). The pH was measured throughout shelf-life and a storage (display day) effect was found ($P < 0.001$). As storage increased, with pH values for all treatments decreasing with storage with mean values ranging from 5.56 on day 0 to 5.25 by day 28 (Fig. 2a). This decrease in pH values with storage is in line with previous work by this group (Van Rooyen, Allen, & O'Connor, 2016). The decline in pH which was observed in this study has also been reported to be related to discolouration (Renerre, 1990).

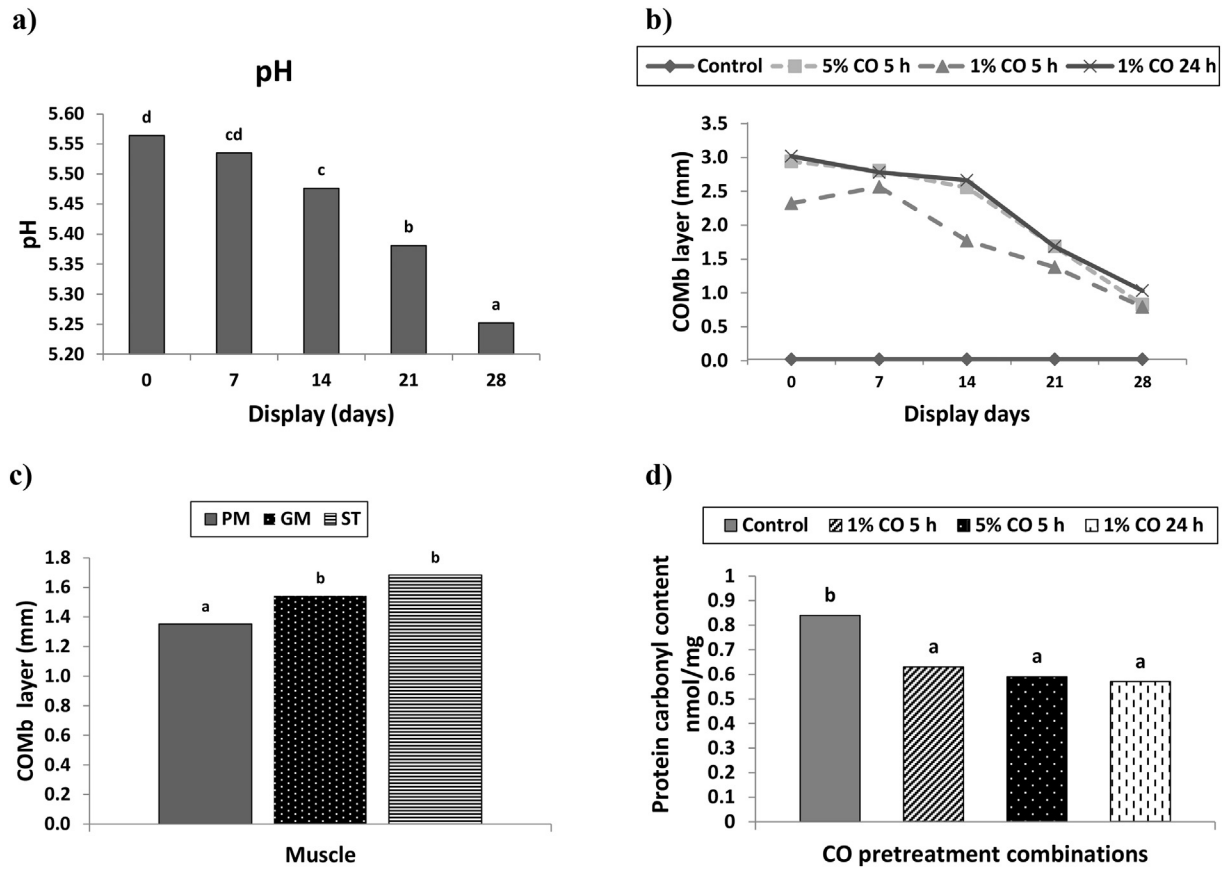


Fig. 2. (a) Effect of display days on pH of steaks stored at 2 °C (averaged for each muscle). Least square means without a common letter are different ($P < 0.05$). Pooled standard error of means (S.E.M) = 0.02 (b) Effect of CO pretreatment and display days on the depth of the carboxymyoglobin (COMb layer) stored at 2 °C (averaged for each muscle). Least square means without a common letter are different ($P < 0.05$). Pooled standard error of means (S.E.M) = 0.17 (c) Effect of muscle type on the depth of the carboxymyoglobin (COMb layer). PM = *Psoas major*, GM = *Gluteus medius* and ST = *Semitendinosus* steaks stored at 2 °C. Least square means without a common letter are different ($P < 0.05$). Pooled standard error of means (S.E.M) = 0.07 (d) Effect of CO pretreatment on carbonyl content (nmol/mg) (averaged for each muscle) stored at 2 °C. Least square means without a common letter are different ($P < 0.05$). Pooled standard error of means (S.E.M) = 0.066

3.3. CO penetration depth

Muscle had an effect ($P < 0.001$) on the depth of the COMb layer (Fig. 2c) with the colour labile PM having the smallest COMb layer while ST had the greatest in line with C* values and a* values on day 0 (Fig. 1c & g). There was a CO pretreatment combination effect × display day interaction ($P < 0.001$) for COMb layer (Fig. 2b). The 1% CO for 24 h treatment had the deepest COMb layer from day 0 to day 28 display was 1% CO for 24 h, while 1% CO 5 h had the shallowest penetration depth compared with the controls (untreated vacuum packaged). The depth of the COMb decreased (< 1 mm) over storage as colour intensity diminished by day 28 corresponding to a* and C* values (Fig. 1b & f). This result is in agreement with previous results by this group (Van Rooyen, Allen, Gallagher, & O'Connor, 2018) and confirms that CO does not mask meat spoilage as the colour intensity diminishes by day 28 of display. The optimum CO pretreatment of 1% CO for 5 h which initially induced colour stability discoloured over storage and had the shallowest COMb layer (0.77 mm) by the end of shelf-life, therefore not masking spoilage. Previous researchers have also reported similar discoloration patterns for CO pretreatments discolouring over storage (Jayasingh, Cornforth, Carpenter, & Whittier, 2001; Sakowska, Guzek, Glabska, & Wierzbicka, 2016). A possible explanation for this trend of COMb layer diminishing over storage could be due to the loss of CO which is bound to the 6th ligand of myoglobin which is lost over display (Hunt et al., 2004).

3.4. Protein carbonyl content

Protein carbonyls are used as an index of protein oxidation (Estevez & Luna, 2017). Protein oxidation was measured at the end of display (Fig. 2d). There was a CO pretreatment effect ($P < 0.01$) with CO pretreatments having lower protein oxidation compared to the controls. All CO preteated samples were lower than the control (Fig. 2d). These results indicate that CO per se has the ability to delay the onset of protein oxidation compared to vacuum packaging. A possible explanation for this may be that CO being an antioxidant acts as an enzyme inactivator thus decelerating myoglobin oxidation, the principal pigment in meat which can induce other oxidative reactions (Besser & Kramer, 1972; dos Santos, Donado-Pestana, Francisquine Delgado, Ossamu Tanaka, & Contreras-Castillo, 2015). Reduced protein oxidation has been reported to be linked to increased tenderness, shelf-life extension, and promotion of enzymatic activity responsible for proteolysis (Lund, Hviid, & Skibsted, 2007; Rowe et al., 2004; Tørngren, 2003). Previous studies have shown that high-oxygen packaging can increase protein oxidation compared to oxygen deficit environments (dos Santos et al., 2015; Fu et al., 2017; Lund, Hviid, & Skibsted, 2007; Zakrys, Hogan, O'Sullivan, Allen, & Kerry, 2008). Therefore it was expected in this study that protein oxidation would be relatively low as conditions were anoxic. A study carried out by dos Santos et al. (2015) showed decreased protein oxidation in beef striploin steaks stored under anaerobic conditions, vacuum or CO-MAP (0.2% or 0.4%), compared to high oxygen MAP. While steaks stored in the highest CO-MAP concentration (0.4%) exhibited the lowest protein oxidation at the

end of shelf-life. However there were no reported differences when anoxic packaging was compared. Similarly, Yang et al. (2016) reported that vacuum packaged and CO-MAP (0.4%) *Longissimus lumborum* (LL) muscles had significantly lower carbonyl content compared to high-oxygen MAP beef steaks. However there were no differences between CO-MAP and vacuum packaged steaks. In contrast to vacuum packaging, the results of this present study show that CO has the ability to inhibit protein oxidation. We therefore conclude that CO is specifically responsible for the reduction in protein oxidation and that this is not due to the oxygen deficit environment. Protein oxidation in this study was relatively limited in all treatments at the end of storage as carbonyl content values were < 1 nmol/mg (Fig. 2d). Lund, Lametsch, Hviid, Jensen, and Skibsted (2007) considered < 1 nmol/mg to be non-oxidised using a slightly different method from that used in this study. Carbonyl content values which range between 2 – 14 nmol/mg are considered to be oxidised and are dependent on initiators of oxidation, muscle type, protein solubility and the degree of oxidation (Lund, Lametsch, et al., 2007). Conversely, muscle (PM, GM or ST) had no effect on protein oxidation ($P > 0.05$). In contrast, previous authors have reported large variability in carbonyl content between different muscle groups (Fagan, Sleczka, & Sohar, 1999; Lund, Lametsch, et al., 2007). A possible explanation for the lack of variability between muscles on protein oxidation may be attributed to the depletion of endogenous antioxidant ability within each muscle tissue due to the prolonged ageing period (as protein oxidation was sampled at the end of storage (35-days post-mortem))(Fu et al., 2017).

3.5. Microbiological analysis

Muscle or gas combination had no effect ($P > 0.05$) on microbiological data for anaerobic mesophiles (TVCm), anaerobic psychrotrophiles (TVCp) and lactic acid bacteria (LAB) (Table 3). This result is in line with previous work by this group (Van Rooyen, Allen, Crawley, and O'Connor (2017) as CO per se showed no microbial inhibition effect. However, CO has been reported to have a bacteriostatic effect using levels of above 5% CO (Gee & Duane Brown, 1981). Since the concentrations of CO used in this present study were not above 5 %, no bacteriostatic effect was observed. All bacterial counts enumerated were considered not spoiled and within the acceptable threshold (7-8 CFU/g) (Table 2.) (FSAI, 2014). One possible factor contributing to discoloration is bacterial proteolytic enzymes attacking globin in the porphyrin and as a result the protein denatures (Renerre, 1990). However CO treated meat products have been reported to retain the bright red appearance even though the microbial population exceeds the levels safe to consume (Sebranek, Hunt, Cornforth, & Brewer, 2006). However this was not the case in this present study, as discoloration occurred for the optimum CO pretreatment of 1% CO 5 h by day 28 and bacterial counts were just below the acceptability limit (FSAI, 2014). In summary, applying CO pretreatments to three muscles differing in colour stability (PM, GM and ST) does not mask meat spoilage.

4. Conclusion

This present study shows the flexibility of CO-pretreatments prior to vacuum packaging and demonstrates that CO pretreatments can be applied to other muscles including *Psoas major* (PM), *Gluteus medius* (GM) and *Semitendinosus* (ST). This study showed CO-pretreatments can improve redness in colour labile steaks including PM and GM over storage. CO pretreatments inhibited protein oxidation compared to vacuum packaged steaks suggesting CO per se is responsible for decreased protein oxidation and is not attributed to the anoxic environment. The optimum CO pretreatment to enhance surface colour while allowing colour to diminish over storage ($a^* = 12$, $C^* = 16$) as to not mask spoilage was 1% CO for 5 h exposure. More importantly 1% CO for 5 h exposure did not mask meat spoilage in any muscle as microbial populations did not exceed the spoilage threshold of (7-8 CFU/g) (Lavieri & Willimas, 2014), so the principle of CO not masking spoilage applies to muscles of differing colour stability. Sebranek et al. (2006) raised a valid point that CO meat packaging systems could create opportunities to improve meat safety. This study strongly agrees with this statement as not only is colour enhanced, protein oxidation is reduced, improving eating quality with no negative effect on spoilage bacteria.

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Further reading

Directive No 95/2/EC (1995). *Of the European Parlament and of the Council on Food Additives Other than Colours and Sweetners*.