



UNIVERSITÀ DEGLI STUDI DI MILANO

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**Effects of oxygen partial pressures
on chemical and biochemical properties of red meats**

AGR\15 and BIO\10

Eleonora Uboldi

Tutor Prof. M. Ambrogina Pagani
Co-Tutor Prof. Sara Limbo
 Prof. Stefania Iametti

PhD course coordinator Prof. M. Ambrogina Pagani

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0. Abstract

Meat is an essential component of a balanced diet, since it is a source of important nutrients totally or almost absent in all other foods. The distribution and sale of fresh meat products present many critical points, and a major concern is the product waste due to uncorrected practices at all levels (i.e. farmer, slaughter house, processor, distributor, retailer, consumer) and short shelf lives. In addition to good manufacturing practices and consumer education, meat packaging can help in prolonging the shelf life of meat products. In fact, the possibility to determine and control the oxygen partial pressure in the environment surrounding the product is the key factor which influence the maintenance of meat quality and safety.

This PhD project aims to evaluate the effect of different oxygen partial pressures on chemical, biochemical and nutritional properties of a variety of fresh red meat products. We selected three specific products: ground beef patties, because their ease of degradation due to the grinding process and the presence of a high quantity of fats; slices from beef meat, because of the homogeneity of their surface; and slices from horse meat, because of its high oxygen sensitivity.

As regarding ground beef patties, a low oxygen solution was studied in deep, inserting case ready units (i.e. foamed trays wrapped with a stretch film) in barrier master bags containing a low oxygen modified atmosphere and oxygen scavengers. After the storage at very low oxygen concentration, patties were exposed to atmospheric oxygen (around 20.7 %) and underwent a display life in air for 2 days. The evolution of chemical and biochemical quality of patties stored in anoxia was compared with that of patties exposed to air for the whole storage. Concerning the anoxic packaging solution, we proved the necessity to select proper primary package materials (i.e. tray, stretch film) to allow the correct gas exchanges between the internal and external side of the package. The selection of high permeable stretch films and open-cells trays allowed not only better reduction of patties' surface pigments during the storage in low oxygen environment, but also better re-oxygenation and colour stability during the display life. Second, the combination of low oxygen storage and display life in air was optimized, and suitable storage and reoxygenation times were identified. The absence of oxygen during the storage resulted in the preservation of both the lipid and protein fraction from oxidation, and the products could be subsequently exposed to air undergoing a 2-days aerobic display life.

Slices from beef meat were the second product selected for evaluation, since the muscle structure was not destroyed by the grinding process, and no evident fats were present. First, a comparison between chemical and biochemical quality evolution during low oxygen storage or traditional exposition to the air was carried out. All chemical, biochemical and microbiological indexes showed the benefits of the low oxygen solution, that contributes in the prolongation of meat shelf life, and especially preserves myoglobin functionality. Second, the optimized low oxygen system was compared with a high oxygen solution. In this case, we focused on the protein pattern evolution and their susceptibility to digestive enzymes. Meat stored in the presence of low oxygen partial pressure showed the higher colour stability for the longer storage time, as well as it registered better biochemical and nutritional properties.

Finally, a preliminary study on slices from horse meat was carried out. This product requires very low oxygen concentration during the storage because its high myoglobin content makes it markedly sensitive to oxidation by free oxygen. Hence, we performed a comparison of different low oxygen solutions on the chemical and biochemical properties of horse meat. Further investigation is needed to evaluate the effects of this storage on protein pattern changes and digestibility.

1. Preface

Meat is the result of several physical and chemical modifications affecting muscle metabolism and structure after slaughtering (Zamora et al., 1996). In fact, the switch to anaerobic metabolism causes a pH decrease and a slower ATP production, from which ensues the difficulty to remove calcium ions (Ca^{2+}) from troponin (*rigor mortis*), and the release of lysosomal proteolytic activities. Rigor mortis decay consists in proteolytic events, lipid oxidation and flavouring production (e.g. acetaldehyde, diacetyl), that provide meat tenderness, colour and flavour. During the shelf life these reactions don't stop: meat aging carries on when meat is exposed in retail shelves, especially because oxygen, temperature and light induce oxidations and microbial growth.

Meat is an essential constituent of a balanced diet, because of its role in human health and development, thanks to the fact that it is either the only source or has a much higher bioavailability for some micronutrients (Biesalski, 2005). As an example, vitamin B12 and A, and iron can be mentioned. In fact, significant amounts of vitamin B12 (cobalamin) and A (retinol) have been detected in animal foods, while vegetable foods don't contain the former (that moreover does not have a provitamin) and provide low amount of β -carotene. This latter is vitamin A provitamin and its conversion efficiency is very low (1:12). Differently, iron sources comprehend animal and vegetable foods, but their bioavailability is definitely higher in the former as it is in a heme form, which is easily absorbed as an intact molecule in the intestinal lumen by enterocytes (Hallberg & Hultén, 2000). The deficiency of one or more nutrients, due to diet poor in animal foods, negatively reflects on human health. For example, vitamin B12 deficiency is correlated to several pathologies, with mild (e.g. lethargy, forgetfulness) to severe symptoms (e.g. megaloblastic anemia and neuropathy) (Pawlak et al., 2001). To reach the suggested intakes of these nutrients, vegetarians and vegans – which have poor or null animal foods intake – may assume supplements or fortified foods, but considering that the bioavailability is generally lower than in foods naturally containing them.

Low intakes of important nutritional facts in a supposed balanced diet can be due to the quality of the food consumed. In fact, the same animal product may present different nutrient concentrations depending on the origin of the animal itself (e.g. breed, weight at slaughter, gender) and on the rearing system adopted. Many authors reported the influence of animal farming, slaughtering and processing protocols and practices on the final quality of meat products. As regarding vitamin content, only Duckett et al. (2009) investigated the effect of grass/forage versus grain finished beef: they reported about twice the riboflavin and three times the thiamine in beef from grass-finished cattle. Furthermore, they reported an increase in antioxidant components (i.e. α -tocopherol and β -carotene) in beef meat thanks to grass-finishing of cattles. Many other authors focused on the lipid fraction different meat species and cuts. Luciano and coauthors (2012) found that pasture-based diets give to the meat a greater oxidative stability compared to diets based on concentrates only, thanks to the higher presence of antioxidants (Wood & Enser, 1997). The main limitation of a totally pasture grazing is the unavailability of grass for extended period in many countries. Hence, later they investigated the effect of pasture and grain feeding combinations on lamb meat oxidative stability (Luciano et al., 2014), reaching the conclusion that the consumption of grass at pasture had a positive effect on lipid oxidation, while a concentrate-based diet greatly increased the susceptibility of lipids to oxidation, independently of the duration of this kind of finishing period. In addition, pasture feeding had a positive effect on sensory quality of meat from calves, since it improved meat texture, as well as more juiciness and flavour, with increasing slaughter weight (dos Santos et al., 2013). Van Elswyck and McNeill (2014) reviewed the US experience regarding the impact of different finishing on beef nutrients and sensory quality, concluding that grass/forage feeding results in leaner cuts with respect to grain finishing, which moreover provides lower amounts of conjugated linoleic acid (CLA).

Not only the graving method, but also behavioural conditions before and during slaughtering influence meat quality. In fact, transportation to slaughter houses is a source of stress, especially when long journeys are required, with negative consequences on animal welfare and meat quality (Knowles et al., 1999). For this reason, in many cases a lairage period is provided to animals so they have sufficient space to lie down, time for recovery, and water and feed available, to restore before slaughtering (Jongman et al., 2008). The extent of lairage needs to be adjusted depending on the length of the journey, in order to prevent mainly dark-firm-dry (DFD) meat and lower slaughter weight, respectively reducing meat loss and reducing the environmental impact of livestock farming (Teke et al., 2014).

After slaughtering, handling and processing procedures are essential in order to ensure the correct transformation of the muscle into meat and maintain the product safety. Besides hygienic protocols essential to avoid cross contamination, de-boning and hanging technique, as well as carcass chilling rate and aging temperature and time affect meat quality (Sorheim & Hildrum, 2002). In the case of hot-boning, an electrical stimulation of carcasses is performed in order to reduce toughening due to sarcomere shortening caused by rapid cooling in pre rigor muscles (Waylan & Kastner, 2004; Kim et al, 2013). In fact, also Wheeler and Koohmaraie (1999) reported that shortened sarcomere reduce the available sites for myofibrillar proteolysis, enhancing meat toughness. This phenomenon can be overcome applying also a stretching treatment right after hot boning (Pen et al., 2012). The investigation of the effects of different aging types resulted in an uncertain contribution of vacuum or dry aging process on sensory properties of meat (Dikeman et al., 2013), while it is perfectly known that the aging process itself contributes to meat tenderization. In fact, the proteinase systems, especially calpains, degrade the structure of myofibrils enhancing the tenderness of the final product. Furthermore, their activity is strongly influenced by temperature, pH and ionic strength and oxidative and nitrosylation status of the proteins in the cell (Huff Lonergan et al., 2010). The degree of meat tenderization is associated with chemical properties of the product which are strictly correlated with consumer acceptability at the time of consumption as well as the digestion efficiency, thus they have a great impact on human diet and nutritional status.

The most important appealing feature of the meat for customers is its appearance and in particular its colour. This is affected by iron in heme molecule properties, especially of myoglobin, pH value, physical characteristics of muscle, lipid oxidation, microbial growth and deteriorative reactions (Mancini & Hunt, 2005). Myoglobin is the main protein involved in meat changes, both organoleptic and chemical. The chemical status in which myoglobin is present in the meat determines its colour: OxyMyoglobin (OMb) gives a bright red, DeoxyMyoglobin (DMb) a purplish red, and MetMyoglobin (MMb) a red-brown colour (Feldhusen et al., 1994). The conversion into these forms is mainly caused by oxygen availability and by proteolytic or denaturing events that change myoglobin structure.

Among all foodstuffs, fresh products are the most sensitive to spoilage and decay, so they are generally characterized by a short shelf life. As a consequence, many packaging systems have been developed in order to improve the shelf life of these products, while preserving their quality and safety (Galić et al., 2011; Kerry et al., 2006; Yam et al., 2005). One of the best examples is the presence of a wide variety of packaging systems currently used in the fresh protein sector. Packaging techniques which could increase meat shelf include Vacuum Packaging, Modified Atmosphere Packaging (MAP), and Active Packaging (based on the release or capture of specific substances) (Piergiovanni & Limbo, 2010; Phillips, 1996).

Since oxygen is one of the key factors for the maintenance of meat quality, MAP solutions are generally classified into high- and low- oxygen systems (McMillin, 2008). The former contains high percentages of oxygen (70-80%), ensuring the saturation of meat pigments and the prevalence of the oxygenated form of myoglobin (OxyMyoglobin, OMb) - which gives a bright-red colour to

the meat -, but also increasing the incidence of oxidative changes, such as lipid oxidation and off-flavours development. Differently, low oxygen systems result in the absence of oxygen (or the presence of very low concentrations not detectable by common gas analysers) and allow the deoxygenation of meat pigments, giving a purple-red colour to the products, generally not appreciated by the consumers.

From a microbiological point of view, under oxygen-depleted atmospheres and $\text{pH} < 5.8$, the growth of facultative anaerobic Enterobacteriaceae is inhibited, and lactic acid bacteria predominate the microflora (Gill, 1996). The storage in ultra-low oxygen packaging and the use of carbon dioxide maintain the microbiological quality also during the following display life in air, thanks to the residual effect of CO_2 (Bingol & Ergun, 2011; Gill & Tan, 1980). The microbial population plays an important role in colour changes on the surface of meat. In fact, the principal role of bacteria in the oxidation of myoglobin (and relative colour change) is due to the growth of aerobes that consume oxygen on the surface layer, preventing further oxygen penetration into tissue and decreasing its partial pressure, thus reaching values which promote myoglobin oxidation (Robach & Costilow, 1961). As a consequence, the level of oxygen inside the packaging during the entire storage is a critical factor that has to be strongly controlled.

The overall equilibrium of the system, which relays on the redox state of meat, is generally guaranteed by the glutathione system: its reduced and oxidized forms ratio is the key for the maintenance of a reducing environment in meat products (Dröge et al., 1994; Miller et al., 2002; Tanimoto et al., 2011). The reducing ability of the system is essential to ensure the formation and maintenance of myoglobin in its reduced and deoxygenated form (DeoxyMyoglobin, DMb) during the storage in low oxygen partial pressure. In fact, at very low oxygen partial pressure (obtained by the use of MAP system also coupled with oxygen scavengers solutions) the deoxygenation of myoglobin occurs through the formation of its oxidised form (MetMyoglobin, MMb), which reduction relies on reducing enzymes from the cytochrome system, and the presence of specific cofactors (Arihara et al., 1995; Bekhit & Faustman, 2005; Young & West, 2005; Tang et al., 2005a). This phenomenon is called transient discoloration and its resolution enables the success of low oxygen packaging systems.

In the present work, we studied the effects of low oxygen partial pressure storage systems on the quality of beef meat slices and patties, and horse meat slices, which represent typical challenges in this field. In the case of beef meat slices, we also evaluated the effects of a high oxygen packaging system, in comparison with the low oxygen one. In particular, low oxygen partial pressure systems were optimized, considering macroscopic indicators (e.g. colour) as well as microbiological evaluation, lipid oxidation and myoglobin forms monitoring. Two main phenomena involving myoglobin forms interconversion were identified and further investigated. Then, a comparison between low and high oxygen partial pressure on sliced beef was carried out, considering also protein modifications and meat digestibility. Finally, as a preliminary work, horse meat behaviour using low oxygen concentration was evaluated, comparing different packaging solutions.

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2. Aim

The aim of this PhD project was to study the effect of oxygen partial pressure on fresh meat quality, taking into consideration chemical and biochemical modifications occurring during storage. The focus was specifically on the application of modified atmosphere packaging solutions for the preservation of red meats.

The selection of red meats aimed to identify a representative scenario of products generally sold at retail level. In particular, the following products were considered:

- Slices of beef meat;
- Patties of ground beef meat;
- Sliced horse meat.

Three different conditions were investigated in different combinations for each meat product: low (<2%), medium (<20 %) and high (<65%) oxygen partial pressures.

During the storage, the evaluation of meat changes was carried out, especially from a chemical and biochemical point of view, and a comparison between different oxygen partial pressures applied to each product was performed.

In the first part of the work the evaluation of ground beef properties during storage in low oxygen partial pressure, using a modified atmosphere packaging system, will be presented. A MAP system coupled with active devices (i.e. oxygen scavengers) will be evaluated: case-ready meat is stored in a low oxygen environment, inside barrier master bags, during handling and back store operations, then it is exposed to atmospheric oxygen (before being positioned onto display cases) and undergoes pigments reoxygenation – the blooming phase. First we aimed to identify the primary package materials, in terms of gas permeability, that were more suitable to ensure the correct gas exchanges needed for the maintenance of meat qualities. Second, the effect of the exposition to the product to low oxygen environments was investigated to optimize a particular packaging solution, with a focus on the kinetics of oxygenation (blooming) after the anoxic storage, as a function of oxygen transmission through the stretch film previously selected. Then, we aimed to deepen the effects of low oxygen partial pressures on meat quality, and sliced beef meat was identified as a reference product, thanks to the homogeneity of its appearance and the absence of evident fat. The main phenomena observed during the testing of patties were further investigated, especially from a biochemical point of view.

The second part will focus on the comparison of beef meat slices chemical and biochemical quality evolution when exposed to high and low oxygen partial pressures, evaluating macroscopic changes as well as protein susceptibility to digestive enzymes.

Finally, we focused on the application of low oxygen partial pressures to horse meat, that is a very challenging product, since it requires strictly anaerobic environments for its preservation. Different systems were tested

The goal of a MA packaging systems is to ensure not only the safety and the great visual appearance of meat, but also the maintenance of some biochemical and nutritional traits during storage. Unfortunately, the lack of scientific knowledge about the effects of packaging systems on chemical, biochemical and also nutritional values of fresh meat led to close examinations, reported in the relative sections of this PhD work.

3. Low oxygen partial pressures: Effects of primary package materials on ground beef patties quality.

3.1 Introduction

In a recent document, the Food and Agriculture Organization of the United Nations has reported on the high levels of foods waste and losses along the supply chains. Meat and meat products do not escape to this phenomenon, and the distribution and consumption represent critical stages both in industrialized and developing countries, making up approximately half of total meat losses and waste (Gustavsson et al., 2011). At these two levels of the food supply chain, the packaging technologies can really play a pivotal role (Wikström & William, 2010). In fact, not only the adoption of new materials, new processes and new storage technologies, but also the optimization of the consolidated counterparts could significantly reduce food losses, better fulfilling consumers' needs, keeping the food fresh longer, and also enhancing the coordination among stakeholders in the supply chain. From this perspective, the shelf life extension strategy for meat and meat products becomes a real mandate. Nowadays, different packaging solutions for the consumer sales unit are available and the case-ready methods (i.e. products not repackaged in the backroom of the store) represent a new paradigm for the shelf life extension of red meat, particularly beef. In addition, labour costs and limited availability of skilled workers at retail locations will continue to drive the demand for case-ready packaging innovations (Eilert, 2005).

Retailers continue to offer the majority of beef meat cuts continue to be offered in a high oxygen environment (approximately 80% oxygen) in order to maintain bloom, with at least 20% carbon dioxide to prevent microbial growth and using a high barrier primary package. Low oxygen packaging systems have been readily available for usage in some countries, but not as widely implemented as the high oxygen counterparts (Eilert, 2005). In fact, most of these systems are not totally adapted to the market needs and realities of each country, thus their potential is not completely expressed.

The object of the present study is to provide support for extended use of low oxygen methods: it consists of an O₂ depleted master bag that contains multiple gas permeable case-ready trays able to effect the conversion of DeoxyMyoglobin to OxyMyoglobin on the surface of meat when exposed to air. This system is largely used in the United States; especially thanks to the approval of low concentrations of carbon monoxide that compensates for the effects of the oxygen depletion on the colour of beef meat, thus maintaining it in the red CarboxyMyoglobin form. Again, the oxygen depletion is successfully accomplished by the use of oxygen scavengers, which also represent an extensively used technology in some countries, like USA and Japan. On the contrary, the adoption of low-oxygen packaging solutions in Europe is strongly limited because the beef meat is usually displayed in its red OxyMyoglobin colour, and the darkened DeoxyMyoglobin formed by oxygen depletion cannot be compensated for by carbon monoxide as it is forbidden. At the same time, the active packaging technology has only recently found a place in the market thanks to the specific Regulation (EC No 450/2009) on active and intelligent materials intended to come into contact with food.

From a technical point of view, the effectiveness of a master bag solution for red meats using O₂ depleted atmospheres depends on different factors (meat quality and cut, CO₂/mass of product ratio, master bag dimensions, etc.) but the residual O₂ and the permeability of the primary packaging are the determining factors. In fact, if residual O₂ is high enough to overwhelm and exhaust the MetMyoglobin reducing activity (MRA) of muscle (Tewari et al., 2002), a permanent discoloration can occur during the storage in the master bag. Various publications highlight the importance of scavenging oxygen as rapidly as possible from the headspace and the use of highly

reactive O₂ absorbers is a real advantage for this packaging solution (Tewari et al., 2001; Tewari et al., 2002; Venturini et al., 2006; Venturini et al., 2010).

At the same time, if beef meat is to be displayed in its red OxyMyoglobin form, the blooming step is crucial and the role of oxygen permeability of film used in the case-ready unit must be carefully evaluated.

Although ultralow-oxygen packaging systems for the storage of meat cuts followed by blooming through oxygen-permeable film have been studied by several authors (Isdell et al., 1999; Tewari et al., 2002; Buffo & Holley, 2005; Belcher, 2006) further information is essential to exploit this packaging solution, especially if applied to very sensitive products like ground beef.

In this work, a master bag solution with O₂ scavengers for patties of ground beef stored in wrapped trays was studied. The aim was to evaluate the role of gas-permeability of different stretch films and the morphology of foamed polystyrene trays in regulating the gas exchanges during the storage in master bag, blooming, and display life.

3.2 Materials & Methods

3.2.1 Meat cuts

Meat from *Semitendinosus* muscle (average weight 4 kg, pH 5.6-5.8) of pure Italian breed "Piemontese" (age 15-18 months, 9-10 days post mortem) was cut (Cutter K 40, Seydelmann, Germany) and ground using an industrial meat grinder (Model 346SS Manual Feed Grinder, Biro, Ohio, USA) with a 4 mm plate. After grinding, 150 g patties (minimum 90% lean) were formed with a patty-forming machine (Planus 869, CRM, Italy). All of these operations were carried out in the back of a retailer in Milan (Italy).

3.2.2 Packaging system

Ground beef patties were packed in EPS trays (140*270*20 mm), containing 2 patties each, for a total weight of about 300 g. two different trays were used: i) closed-cell EPS trays not perforated with an oxygen permeability at 23°C around 180 cm³ 24h⁻¹bar⁻¹; ii) open-cell EPS trays perforated on the bottom (density of perforation: 9 holes/cm² distributed on 27*7 cm), with an oxygen permeability at 23°C around 1200 cm³ 24h⁻¹bar⁻¹. Also, three different wrapping films were used: i) stretch PVC film with O₂TR of about 20,000 cm³ m⁻² 24h⁻¹ at 23°C, 0% RH and 1 bar of pO₂ (named PVC 20000); ii) PVC film with O₂TR of about 8,000 cm³ m⁻² 24h⁻¹ at 23°C, 0% RH and 1 bar of pO₂ (named PVC 8000); iii) LLDPE stretch film with O₂TR of about 26,000 cm³ m⁻² 24h⁻¹ at 23°C, 0% RH and 1 bar pO₂ (named LLDPE 26000). Four trays were inserted in a barrier master bag (nylon based, 60*43cm, O₂TR 70 cm³m⁻² 24h⁻¹bar⁻¹ at 23°C) with two oxygen scavengers (FreshPax[®] CR 8, Multisorb Technologies). The master bag were sealed in modified atmosphere (30% CO₂ and 70% N₂) using a CVP machine (Downers Grove, IL), applying a double vacuum-flush cycle at the following conditions: vacuum 7 s, flushing 0.8 s for the first cycle; vacuum 6.5 s, flushing 3 s for the second cycle. The sealing time was 2.7 s. Patties were stored in the dark for 10 days at 0.5±0.5°C: master bag were opened after 4, 7 and 10 days, then meat was allowed to reoxygenate (blooming) at 4±1°C and finally exposed onto a display case for 48 hours in order to simulate the display life at retail level. At each opening time, meat was analysed before (BB) and after (AB) blooming, and after 24 and 48 hours of display life.

3.2.3 Methods

3.2.3.1 Headspace analysis

Residual oxygen concentration (%) inside master bags was measured by using a non-invasive oxygen measurement device (OxisenseTM 157 101, DecisionLink Inc., Dallas, TX, USA), applying a photosensitive dot inside master bags before their sealing. The O₂ concentration was measured in five different master bags immediately after packaging (time 0) and during the first ten hours of storage. The minimum detection limit of the instrument was 0.08% O₂ (about 0.8 mbar) at 5°C at 1.010 bar of pressure.

3.2.3.2 Colour evaluation and visual appearance

Colour indexes were recorded after master bags opening, after the blooming and during the display life at 4±2°C. Colour instrumental measurements were carried out with a hand-held tri-stimulus colorimeter (Minolta Chroma Mether CR-210, Minolta, Osaka, Japan) with a 8 mm viewing port, 2° standard observer and a C illuminant source. Before each measurements, the apparatus was calibrated on the Hunterlab colour space system using a white ceramic tile (Minolta calibration plate, Y= 92.6, x= 0.3136, y= 0.3196). Colour was described as Hue angle (H°, expressed as arctg b*/a*) and Chroma (C*, expressed as (a*²+b*²)^{1/2}). The analyses were repeated, at least, on three patties from different trays and each measure was also replicated on the same patty.

Full colour images of patties were acquired at 300 dpi resolution (full colour) with a flat bed scanner CanonScan LiDE 200 (Canon Inc., Tokio, Japan), with a light emitting diode as illuminant, in pre-standardized conditions (black box over imposing) (Riva et al., 2005). Data were stored as PNG and JPG files.

3.2.3.3 pH

The pH value of steaks was measured directly on the beef slices using a membrane glass probe type electrode (Crisolyt A, Crison PH meter GLP 22, Barcelona, ES).

3.2.3.4 Microbiological analyses

The patties were aseptically removed from the trays and 10 g were homogenized in 90 ml of a sterile trypton salt solution at 0.85% into a sterile Stomacher bag, using a Calworth 400 Stomacher for 3 min. Tenfold progressive dilutions were prepared and the following bacteriological determinations were carried out. Mesophilic aerobic bacterial count was assessed by pour plates on Plate Count Agar (Merck Germany) (ISO, 2003), incubation at 30°C for 72 h. Total *Pseudomonas* spp. was assessed by spread technique on Pseudomonas Agar CFC Selective Agar (Merck Germany) (ISO, 2010), incubation at 35°C for 5 d. Lactic Acid Bacteria were measured by pour plates on APT (Merck Germany), incubation at 30°C for 48 h under anaerobic condition (gas pack). *Enterobacteriaceae* were assessed by double layer pour plates on Violet Red Bile Dextrose Agar (VRBD), (Merck Germany) (ISO, 2004), incubation at 37°C for 24 h. *E. coli* was measured by pour plates on TBX (Merck Germany) (ISO, 2001), incubation at 37°C for 24 h. Spores of *Clostridium perfringens* was counted in the presence of Sulphite Polimixin Sulfadiazine after pasteurization at 80°C for 10 min according to Angelotti et al. (1962). *Brochrotix thermosphacta* was assessed in STAA (Merck Germany) by spread plates, incubation at 30°C for 48 -72 h (Dainty & Hibbard, 1980).

3.2.3.5 Myoglobin form estimation

Visible reflectance spectra (of 380-780 nm) were obtained from two different locations of the slice using a UV-visible spectrophotometer (Lambda 650, Perkin Elmer, Italy) equipped with an integrating sphere (110 mm diameter, incident reflectance angle 8°, Perkin Elmer, Italy), with a 1.5 cm diameter viewing port, 2° standard observer, illuminant C. Three different steaks were analysed at each scheduled time taking 3 readings repeated on 2 different positions of each steak. Reflectance spectra were recorded placing the samples onto the reflectance port of the integrating sphere, maintaining the specular door opened. Reflectance values of the different myoglobin oxidation states were estimated at specific wavelengths, and converted to K/S values (K is the absorption coefficient and S is the scattering coefficient). The K/S values were used for quantifying the proportion of OxyMyoglobin (OxyMb), DeoxyMyoglobin (DeoxyMb), and MetMyoglobin (MetMb), calculated using specific wavelengths for fresh meat colour in accordance with the AMSA guidelines (AMSA, 1991). In particular, the ratios and the wavelengths used for the calculus were: $K/S_{474} \div K/S_{525}$ for % DeoxyMb; $K/S_{575} \div K/S_{525}$ for % MetMb and $K/S_{610} \div K/S_{525}$ for OxyMb. The pure forms of DeoxyMb, MetMb and OxyMb were obtained on the fresh slices as proposed by AMSA (1991, 2012) with minor modifications. Reference MetMb form in meat was obtained by placing a piece of steak into 50 ml of 1% potassium ferricyanide for 1 min, followed by draining, surface blotting, and sample wrapping in an oxygen permeable PVC cling film for 12-14 h at 2°C. Immediately after this treatment, the spectrophotometric spectrum was acquired. The reference DeoxyMb form was obtained packing a piece of meat into a small gas barrier pouch flushed with 100% N₂ atmosphere. Through a septum stuck onto the pouch surface, 10 ml of a solution of 10% sodium dithionite were applied directly onto the meat surface with a gas tight syringe. The treated meat was stored for 2 h at room temperature (22±1°C), after which the meat was rapidly removed, drained, and wrapped with a PVC cling film for the scanning with the spectrophotometer. The reference OxyMb form was obtained directly on fresh meat immediately after the preparation of the steaks from the *Semiteminosus* muscle. Within 5 minutes from the opening of the master bag, the sample was cut from a refrigerated piece of meat, wrapped with a PVC cling film and the reflectance measurements were recorded with the spectrophotometer (room temperature 16±2°C).

3.2.3.6 Secondary lipid oxidation – TBA method

The thiobarbituric acid (TBA) assay was performed following the method proposed by Raharjo, Sofos, and Schmidt (1993). Ten g of minced meat were homogenized with 40 ml of 5% (w/v) aqueous trichloroacetic acid (TCA) at room temperature for 1 min. The homogenate was centrifuged at 10,000 rpm for 5 min and the supernatant was filtered through a Whatman micro fiber glass filter (grade C, Whatman, GF/C Intern Ltd Maidstone, England) into a volumetric flask. Filtrate volume was adjusted to 50 ml using 5% (w/v) TCA. A volume of 5 ml was reacted with 5 ml of 80 mM TBA in a test tube with a screw cap while heating in a water bath at 94±1°C for 5 min. The pH was adjusted to 7 with 5 N NaOH and 0.2 ml of 3% (w/v) phosphate buffer, pH 7.2, prior to loading on a solid phase extraction tube (Strata C18-T, 1g/6ml cartridge, Phenomenex, Italy). After cartridge conditioning with methanol and water, 10 ml of the sample were loaded and the eluted solution from the cartridge was discarded. Unreacted TBA solution and other components were removed by eluting the loaded sample with 10 ml of distilled water. The red malondialdehyde-TBA complex was recovered and separated from other thiobarbituric acid reactive substances (TBARS) by eluting the cartridge with 10 ml of absolute methanol. The absorbance of the methanol eluent containing the malondialdehyde-TBA complex was measured at 525 nm in an UV/Vis spectrophotometer (Perkin Elmer Lambda 25, Italy). The calibration curve was obtained from serial diluted solutions of 10⁻³ M TEP (1,1,3,3-tetra-etoxypropane), following

the same procedure. The results were expressed as 2-thiobarbituric acid reactive substances (TBARS) in mg malondialdehyde per kg meat. The analyses were made on three steaks from different trays.

3.2.3.7 SDS-PAGE analysis

Electrophoretic analysis of protein fractions was used to verify qualitatively proteolytic events during storage and shelf-life. Aliquots of lyophilized meat (1 mg) were suspended in 250 µl of water and 250 µl of denaturing buffer (0.125 M Tris-HCl, pH 6.8; 50% glycerol (v/v); 1.7% SDS (w/v); 0.01% Bromophenol Blue (w/v); 1% 2-mercaptoethanol (v/v)), and heated at 100°C for 10 min. After cooling, samples were centrifuged (micro-centrifuge, 10,000 × g, 3 min) and 20 µl of each supernatant were loaded on the gel (12% monomer) in a MiniProtein apparatus (BioRad, Richmond, VA, USA). Gels were stained with Coomassie Blue and destained in aqueous 30% v/v ethanol, 10% v/v acetic acid. The protein MW standard was a cocktail of eight individual marker proteins (MW 14–97 kDa) (Sigma Aldrich, Milan, Italy).

3.2.3.8 Statistical analysis

One-way analysis of variance was used to assess the effect of PVC permeability and EPS structure on Hue angle and Chroma of beef patties at each display time. Differences among the effects were determined by calculation of Fisher's LSD (Least Significant Differences) test when the packaging effect was significant ($p < 0.05$).

Mean values were calculated by the analysis of variance using Statgraphics Plus 4.0 (Statpoint Technologies, Inc., Warrenton, Virginia, USA).

3.3 Results & Discussion

In this work, patties were wrapped in stretch films with different O_2TR values (as listed in section 3.2.2), using the same closed-cell EPS tray and stored in a master bag system, in order to evaluate the effects of the wrapping film permeability on colour stability during the storage inside the master bags, the blooming and the display life.

The complexity of the oxygen exchanges between the headspace of the case-ready unit and the headspace of the master bag during the first hours of storage required the use of oxygen scavengers inside the master bag to remove the residual of oxygen and to avoid meat acting itself as a scavenger, as demonstrated by previous works (Tewari et al., 2002). In fact, the oxygen entrapped in the case-ready tray or in the meat cut, and the oxygen ingress through the master bag over the storage period can cause a non-reversible discoloration of the meat. For example, Gill and McGinnis (1995) showed that red beef meats with low colour stability, such as ground beef, can discolour even when O_2 concentration is below 0.05%.

In the presence of scavengers, the mean residual of O_2 estimated inside the master bags immediately after their closure, was $1.60 \pm 0.30\%$, independent of the primary package used. During the first 10 hours, oxygen scavengers were able to provide a rapid reduction of the concentration of residual oxygen inside the master bags, allowing the establishment of oxygen levels lower than the limit of detection (0.08%) of the instrumental device used to monitor the gas in the headspace (Figure 3.1). This level was maintained over the entire storage period and effectively facilitated the reduction of myoglobin to obtain DeoxyMyoglobin (DMb). In fact, this conversion may occur only

at extremely low oxygen partial pressure, through the formation of reversible MetMyoglobin (MMb) form (Mancini & Hunt, 2005).

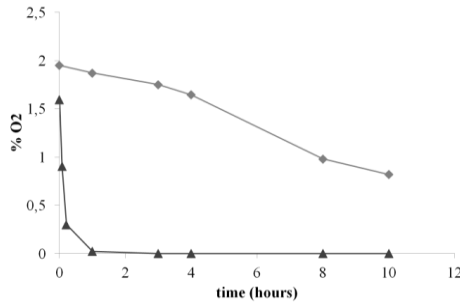


Figure 3.1. Mean evolution of oxygen inside master bags with (▲) and without (◆) scavengers.

In this experiment, the oxygen concentration inside master bags without the oxygen scavengers was around 1.5% and the level did not decrease quickly in the first hours of storage (Figure 3.1). These values compromised the colour stability of the meat surface: after few days of storage the patties changed their colour in an irreversibly, turning the surface towards brown hues (Figure 3.2). For this reason, the experimental plan was carried out considering only the patties stored in master bag with scavengers.

STORAGE	0	7	24 hours	48 hours
a			NA	NA
b		NA		
c				

Figure 3.2. Digitalized images of the patties differently stored: a) wrapped with LLDPE 26000 and stored in master bag without scavengers; b) wrapped with PVC 20000 and never stored in master bag (reference); c) wrapped with LLDPE 26000 and stored 7 days in master bag with scavengers. The images refer at different time of storage: time 0, 7 days after blooming, and 24 and 48 h of display life.

Hue angle (H°) and Chroma indexes were selected as the most representative parameters for colour evaluation of meat (Cassens et al., 2006). In fact, meat purchasing decisions are influenced more by color than any other quality factors (Mancini & Hunt, 2005). In their study, Carpenter and co-authors (2001) highlighted the strong relationship between colour preference and purchase intent by consumers who would discriminate against beef that was not red.

Colour indexes were recorded after master bags opening (7 days), after the blooming (2 hours) and during the display life (24 and 48 hours). After 7 days of anoxic storage in master bag, Hue angle of the upper surface of patties slightly decreased (Table 2, BB), reaching values around 12° in meat stored with the two PVC stretch films: this decrease is associated with the presence of DeoxyMyoglobin (DMb) at higher concentrations (it passed from 8.35±0.37% at time 0 to 36.4±6.3% after 7 days in master bag; data not shown), giving a purple hue to the meat. Statistical differences ($p<0.05$) were found in samples wrapped with the PVC 8000: in this case, Hue angle decreased up to 17°, a value that corresponds to a brownish red. Hence it is possible to assume that, during the storage in master bags, some changes occurred on patties surface, but only the presence of high O₂TR films allowed a stronger deoxygenation, thanks to the higher possible rate of gas exchanges between the tray and the master bag headspaces (Boonruang et al., 2012).

Chroma is a more reliable indicator of meat discoloration because it quantifies greying as an accumulation of white within a pure red colour: in our study, for all the case-ready solutions, an appreciable decrease was observed between samples at time 0 and those stored in anoxia, indicating the change of the saturation towards darker colour, as is the purple-red DMb comparing to the brightest OxyMyoglobin (Mancini & Hunt, 2005).

Storage time	Film	L*	Hue angle (°)	Chroma
0	PVC 8000	45,64±0,39 ^a	21,14±0,71 ^a	30,88±0,17 ^a
	PVC 20000	44,12±0,54 ^b	19,08±0,10 ^b	33,11±0,38 ^b
	LLDPE 26000	44,39±0,75 ^b	19,27±0,25 ^b	32,75±0,53 ^b
7 BB	PVC 8000	45,44±0,26 ^a	16,72±0,42 ^a	28,61±0,49 ^a
	PVC 20000	44,95±0,79 ^a	12,88±0,68 ^b	28,69±0,51 ^a
	LLDPE 26000	44,64±0,32 ^a	12,77±0,85 ^b	29,57±0,62 ^{ab}
7 AB	PVC 8000	46,39±0,21 ^a	19,55±0,03 ^a	29,60±0,37 ^a
	PVC 20000	45,16±0,46 ^b	18,22±0,01 ^b	32,69±0,67 ^b
	LLDPE 26000	45,88±0,36 ^c	19,05±0,11 ^c	35,70±0,36 ^c
7+1	PVC 8000	46,58±0,20 ^a	25,50±0,88 ^a	19,32±0,38 ^a
	PVC 20000	44,37±0,61 ^b	19,75±0,17 ^b	29,10±0,20 ^b
	LLDPE 26000	45,05±0,56 ^c	19,21±0,06 ^b	32,75±0,56 ^c
7+2	PVC 8000	47,37±0,56 ^a	26,99±0,31 ^a	19,39±0,06 ^a
	PVC 20000	44,79±0,33 ^b	21,92±0,21 ^b	24,10±0,34 ^b
	LLDPE 26000	45,35±0,30 ^c	20,89±0,15 ^c	26,09±0,34 ^c

Table 3.1. Evolution of colorimetric (L*, Hue angle and Chroma) values of patties wrapped with different stretch films at time 0, after 7 days of storage in master bags, before (BB) and after (AB) blooming and during display life (+1 and +2 days). In each column, at each time of analysis, different letters indicate a significant difference among samples ($p<0.05$).

After the removal from master bags, patties were stored in their primary packages for two hours in air at 4°C, to allow the myoglobin re-oxygenation (blooming), and then exposed onto a display case, to simulate the display life at retail level. In fact, most red meats are marketed in their

oxygenated form, and the myoglobin in meat stored in low oxygen systems must oxygenate (or "bloom") prior to display (Beggan et al., 2006) to promote OxyMyoglobin (OMb) formation. In the anoxic packaging systems, where the blooming is expected, a crucial point is the oxygen-transmitting ability of the overwrap film. Different Authors (Isdell et al., 1999; Beggan et al., 2004) suggested that a highly oxygen-permeable overwrap film (with OTR values around $20000 \text{ cm}^3 \text{ 24h}^{-1} \text{ m}^{-2} \text{ bar}^{-1}$ at 23°C , $0\%\text{RH}$) allows the achievement of blooming in meat cuts like steaks of *Longissimus lumborum*, while new information are necessary concerning this requirement for ground meats or patties.

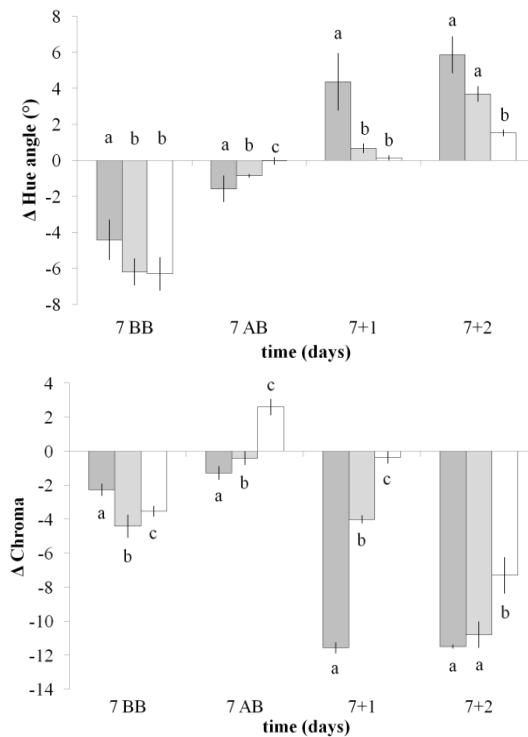


Figure 3.3. Delta Hue angle and Delta Chroma for patties stored in master bag 7 days, before (BB) and after (AB) blooming and during display life (+1 and +2 days). Patties wrapped with PVC 8000 (■), PVC 20000 (▒) and LLDPE 26000 (□).

After the blooming, statistical differences ($p < 0.05$) were noticed among samples stored with the different stretch films, as regarding both Hue angle and Chroma indexes (Table 3.1). The latter was the best indicator of blooming in meat exposed to air (Young & West, 2005). In fact, it better expressed the re-oxygenation of patties as function of the oxygen transmission rates of stretch films. In particular, patties stored with LLDPE 26000, the most permeable film used in the test, showed the highest Chroma value, corresponding to a brighter colour. The balance between oxygen concentration at the surface and tissue respiration, which consumes oxygen as it becomes available, influences the depth to which the oxygen penetrates (Millar et al., 2004). Thus, blooming would be expected to be more efficient under conditions that increase oxygen solubility and discourage

enzymatic activity such as a high-oxygen environment, low temperature and normal pH (Beggan et al., 2006).

To highlight the role of O₂TR during blooming, in Figure 3.3 the results were also expressed as the difference between the Hue angle or Chroma at the time of analysis (H_t or C_t) and the respective value at time 0 (H₀ or C₀).

After blooming the lowest difference in Hue referring to t₀ was detected for the sample stored in LLDPE film. The high permeability of the film allowed the re-oxygenation of the patties that gained Chroma values higher than those measured at time zero: as discussed by Lindahl and co-authors (2001) this is due to the loss of activity of oxygen-consuming enzymes during the storage in anoxic environment. In fact, if the respiratory activity of meat is lowered during storage and the oxygen is not consumed rapidly, not only the blooming occurs more quickly, but also the OxyMyoglobin layer is therefore thicker. This phenomenon always occurs on chill-stored meat displayed under an oxygen permeable wrap (Young et al., 2005). Moreover, patties used in this study were obtained from *Semitendinosus* muscle that has a low oxygen consumption rate, if compared to other muscles, because of the greater α -white fiber content that is characterized by less mitochondria (Seyfert et al., 2007). Also this fact can explain the ability of meat to bloom, as shown by Chroma values that exceeded those at time zero.

The change of Hue angle towards higher values during the display life and the change of Chroma towards lower values correlated to the oxidative phenomena involving meat pigments, and in particular to the formation of MetMyoglobin on the product surface (Mancini & Hunt, 2005). As evident in Figure 3.4, the oxidation of myoglobin was more pronounced in patties wrapped with lower permeability films (as evidenced by the highest changes of colour indexes with respect to time 0), leading to the conclusion that the higher oxygen permeation through the stretch LLDPE seems to have contributed to a longer colour stability of the patties, thanks to the oxygenation of myoglobin for a longer time.

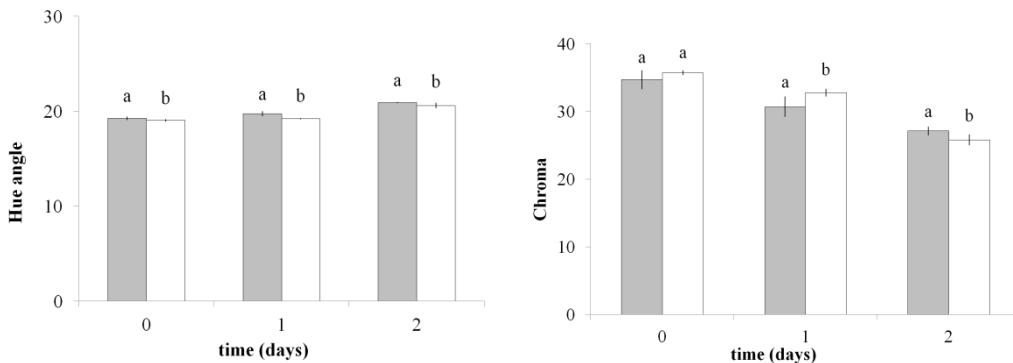


Figure 3.4. Hue angle and Chroma evolution during the display life of patties wrapped with PVC 20000 (■) and never stored in master bag, and patties wrapped with LLDPE 26000 (□) and stored 7 days in master bag.

During 48h of display, meat packed with the LLDPE stretch film (after storage in master bag and blooming), and meat never stored in master bag and packed in air inside a standard EPS tray wrapped with PVC 20000 film were compared (Figure 3). Significant differences ($p < 0.05$) between the control and the samples previously stored in the anoxic bag were detected, at each time of analysis and for both colorimetric indexes (H° and C*). The trend of the colour changes over the

display life was quite similar, though samples stored 7 days in master bag showed a lower rate of H° and C^* indexes changes during the display life, due both to the higher permeability of the stretch film and to the different storage conditions preceding the display life (Figure 3.4).

In general, the storage in master bag did not negatively affect the appearance during the display life of patties wrapped with LLDPE 26000, as evident also from images shown in Figure 3.2.

The microbiological analyses confirmed the quality of the products throughout the whole storage in master bag and the subsequent display life. After 7 days of storage in master bag, the total bacterial count (TBC) reached a concentration of 10^6 cfu g^{-1} (at time 0 it was around $2.4 \cdot 10^5$ cfu g^{-1}), being formed mostly of/from lactic acid bacteria. The acidifying action of the latter, combined with the dissolution of the CO_2 of the modified atmosphere in the product, helped in preventing *Pseudomonas* spp. growth, the most important spoilage microorganisms found in fresh meats (Jay, 1996; McMillin 2008). In fact, its concentration remained constant at around $2 \cdot 10^4$ cfu g^{-1} till the seventh day of anoxic storage.

At the end of the display life, we observed an increase in the TBC of about 2 log and in *Pseudomonas* of about 1 log. On the contrary, in the control samples (patties never stored in master bag but undergone the display evaluation) we registered significantly greater increases of both indexes after only one day of display life: the TBC reached mean values of around 10^7 cfu g^{-1} and *Pseudomonas* spp. of around 10^6 cfu g^{-1} . It is possible to hypothesize that the prolonged anoxic storage and the presence of CO_2 in the MA were able to reduce the vitality of microorganisms, consequently slowing their growth also during the aerobic display life.

The effects of the trays structure on the colour stability of the bottom side of patties, during the storage inside the master bags, the blooming and the display life were also evaluated. Usually, the use of open-cells structure for foamed polystyrene is aimed to absorb the water and water-based juices (i.e. meat exudates).

Some studies highlighted the role of open-cells structure in regulating gas exchanges through the tray, especially when the food product is characterized by high respiration rate, like vegetables (Piergiovanni et al., 1999). In our study, patties were positioned onto two different trays (open-cells and closed-cell, listed at section 5.2.2) and wrapped with the same PVC 20000, before the master bag packaging. Also in this case, Hue angle (H°) and Chroma (C^*) were the selected indexes for describing quality evolution: colorimetric measurement were performed at time zero, after 7 days of storage in master bag, after blooming and during the display life (for specific time and temperature refer to Material & Methods section).

After 7 days of storage in master bag, the difference between samples packed using different trays was statistically significant but not so important. The differences increased after blooming and over the display life, in the over wrapped trays in air for 24 and 48 hours.

Colour indexes indicated a slower change occurring on the bottom side of patties packed in open-cells trays ($p < 0.05$) compared to closed-cell trays (Figure 3.5). We can hypothesise that the structure of the open-cells EPS favoured a higher rate of gas exchange between the external environment and the internal headspace of the primary package (as also indicated by the higher O_2TR value of the tray), and avoided the reaching of critical oxygen partial pressures, which might cause a faster discoloration of the meat pigments. For instance, if the oxygen cannot diffuse rapidly through the meat, the DeoxyMyoglobin is more readily converted into MetMyoglobin.

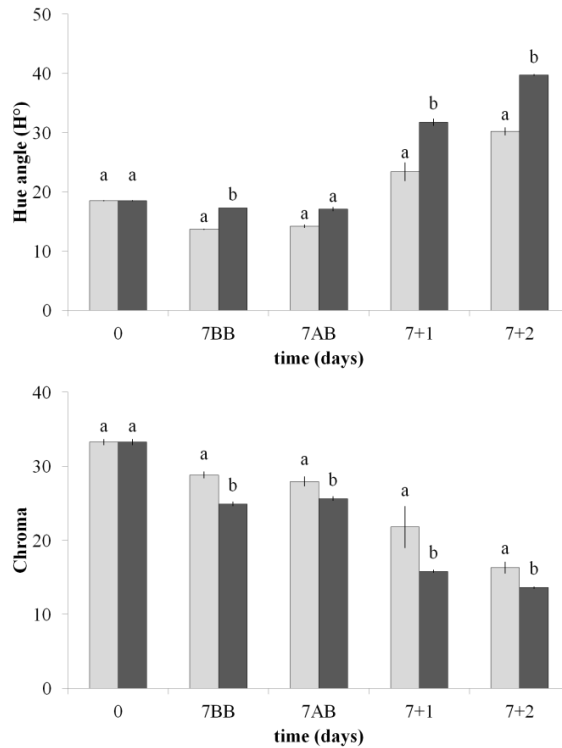


Figure 3.5. Chroma and Hue angle evolution during the master bag storage (7 days), blooming (before BB and after AB) and display life (+1 and +2 days) of patties packed into open-cells (□) or closed-cell (■) trays and wrapped with PVC 20000.

The permeability of the closed-cells tray and its morphological structure influenced the colour changes on the bottom side of patties. In fact, in Figure 3.5 we can observe that after only one day of display life, Hue angle and Chroma of the patties packed with the closed-cell tray differed significantly from the products packed inside the open-cells tray. In particular, patties stored in the former had a brown-red hue (correlated to higher presence of oxidized surface pigments), while in the latter they maintained a bright-red hue.

3.4 Conclusions

This study confirmed the positive effects of a low oxygen master bag packaging system for prolonging the commercial life of ground beef patties. The presence of the oxygen scavengers inside the master bag guaranteed the maintenance of the right atmosphere avoiding the permanent discoloration during the anoxic storage. The re-oxygenation of the meat pigment during blooming is function both of the stretch film O₂TRs' and the morphological structure of EPS trays used as primary packages for patties.

In particular, the more permeable film (LLDPE 26000) allowed not only better reduction of patties' surface pigments during the storage in master bags (7 days), but also better re-oxygenation and colour stability during the two days of display life. In addition, the use of open-cells trays

permitted the least discoloration on the bottom side of patties stored for 7 days in master bag and 2 days in a display case.

We can conclude that the selection of primary package materials, with particular attention to their structure and gas permeability, is a key factor for the optimization of a low oxygen master bag packaging system intended for ground beef patties. In fact, the right performances of materials can contribute to the colour stability of the product both on the upper and lower side of patties, extending their distribution life and making the master bag solution competitive if compared with the simple tray wrap solution.

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4. Low oxygen partial pressures: Chemical and biochemical quality evolution of ground beef patties.

4.1 Introduction

Patties made with ground beef meat are very common products on the shelves of retailer stores, thanks to their ease of use and versatility. Ground meat is generally more perishable than whole or sliced meat because the cutting and grinding processes expose the deeper sterile tissues to atmospheric oxygen and because nutrients escape from the cells, thereby becoming available for microorganisms. Moreover, the greater exposed surface area enhances oxidative and degradative reactions. For these reasons, ground meat is generally classified as one of the most hygienically risky foods and a good habitat for food-poisoning microorganisms that are dangerous to humans (e.g. *Listeria monocytogenes*) (Lund et al., 2000; Noriega et al., 2010).

The selection of the packaging solutions plays an important role in protecting ground meats; it has to guarantee the safety of the product while simultaneously promoting longer shelf life and reducing food waste, especially at retail and consumer levels. Different packaging systems can be chosen when distributing ground beef patties. The most common solution consists of a case-ready unit made of non-barrier materials, such as expanded polystyrene trays (EPS), which are usually overwrapped with stretch films possessing very high oxygen transmission rates (O_2TR higher than $10,000-15,000 \text{ cm}^3 \text{ m}^{-2} 24 \text{ h}^{-1}$ at 23°C). The shelf life of these tray-wrapped patties, if stored at 4°C , is not longer than 3 days in display cases at retail stores (John et al., 2004).

To improve the redness stability of patties, the “tray lidding” packaging system can be used, which consists of barrier trays sealed with a barrier film. The bright red colour associated with freshness is obtained by packaging the meat in a modified atmosphere (MA) containing up to 80% O_2 with the remainder as carbon dioxide (CO_2) (Jayasingh et al., 2002). The shelf life of patties can be prolonged for 10 days or more, but the high oxygen levels promote rancidity (Jayasingh et al., 2002; Smiddy et al., 2002; Limbo et al., 2010) and premature browning (Hunt et al., 1999; Grobbel et al., 2008a) and may have a negative impact in the sensory quality of meat after cooking, especially in terms of juiciness and flavour (Grobbel et al., 2008b; Zakrys-Waliwander et al., 2010).

To reduce the oxidative reactions in beef meat, low-oxygen packaging systems are used, such as vacuum packaging and low-oxygen MAs. The distribution life in the low-oxygen MA state is extended by 5-10 days depending on the cut and the headspace of the package, which must be minimized as much as possible to avoid residual oxygen. The drawbacks of this packaging solution consist of the colour change of the beef meat (purple hues are not appreciated by consumers at the purchasing level) and the risk of permanent discoloration caused by low oxygen residuals.

The aim of this work was to study the effects of a master bag low-oxygen packaging system on ground beef patty quality. This system seeks to extend the quality of the product by distributing it in the purple state and then marketing the product in the bright red state. Specifically, it consists of the insertion of multiple case-ready units of patties packed with non-barrier materials inside a large barrier master bag containing oxygen scavengers. After being filled, the atmosphere in the master bags is vacuumed and then flushed with the desired MA, sealed and stored at approximately $0-1^\circ\text{C}$. Before being displayed on shelves (display life), the product is placed into contact with atmospheric oxygen, which causes it to bloom and thus achieve the bright red colour typical of fresh meat that is familiar to consumers.

The evolution of quality indexes during storage, blooming and display life will be described and discussed with a focus on the kinetics of oxygenation (blooming) after the anoxic storage as a function of oxygen transmission through the stretch film.

4.2 Materials & Methods

4.2.1 Meat cuts

Refer to section 3.2.1.

4.2.2 Packaging systems

Ground beef patties were provided from a local grocery store already packed into expanded polystyrene (EPS) trays (27*14*2 cm) and overwrapped with a stretch PVC film with an O₂TR of approximately 22,000 cm³ m⁻² 24 h⁻¹ at 23°C, 0% RH and 1 bar of pO₂. Each tray contained 2 patties for a total weight of approximately 300 g. The headspace of the tray was reduced as much as possible, and the stretch film was in direct contact with the upper surface of the patties.

Scheduled analysis time	Number of master bags (MBs)	Number of trays
4 days of storage in MB	11	44
- <i>Before blooming</i>		4 (UP)*
- <i>Blooming kinetics</i>		32 (16 UP+16P)* °
- <i>Display life</i>		8 (UP)
8 days of storage in MB	11	44
- <i>Before blooming</i>		4 (UP)*
- <i>Blooming kinetics</i>		32 (16 UP+16P)*
- <i>Display life</i>		8 (UP)
10 days of storage in MB	11	44
- <i>Before blooming</i>		4 (UP)*
- <i>Blooming kinetics</i>		32 (16 UP+16P)*
- <i>Display life</i>		8 (UP)

*UP= unperforated PVC film; P=perforated PVC film.

° Blooming kinetics: total number of trays used to describe the kinetics of blooming. At each blooming time, 8 trays (4UP+4P) from different MBs were used.

Table 4.1. Number of master bags and trays used in the experimental plan

Four trays were inserted inside a master bag (60*40 cm) of coextruded material (PE/EVOH/PE) with an O₂TR of less than 1 cm³ m⁻² 24 h⁻¹ at 23°C and 1 bar pO₂. The master bag packaging was performed with the use of a CVP machine (Downers grove, IL), applying a double vacuum-flush cycle under the following conditions: vacuum 7 s, flushing 0.8 s for the first cycle; vacuum 6.5 s, flushing 3 s for the second cycle. The sealing time was 2.7 s. The composition of the atmosphere inserted into the master bag was 30% carbon dioxide (CO₂) and 70% nitrogen (N₂). Additionally, two iron-based pre-activated oxygen scavengers (FreshPax[®] CR, Multisorb Technologies, nominal capacity 800 cm³) were inserted inside each master bag before sealing.

A total of 132 trays divided into 33 master bags were prepared as described in Table 4.1.

The master bags were divided into three batches to perform analyses after 4, 8 and 10 days of storage. All the master bags were maintained in the dark at $0.5 \pm 0.5^\circ\text{C}$, and when each master bag was opened, the blooming phase and display life were monitored for 0.5, 1, 3 and 5 hours at $3 \pm 1^\circ\text{C}$ and for 48 hours at $4 \pm 2^\circ\text{C}$. To evaluate the blooming phase, after the master bag was opened, the PVC stretch film of half of the packages was manually perforated using a toothed wheel to allow free atmospheric exchange during the blooming time. Each film had 12 holes with a 0.36 ± 0.07 mm diameter for a total perforated area of 0.407 mm^2 (density of perforation 1 hole/40.5 cm²). The hole sizes were verified through optical microscope analyses carried out on 10 samples of film after wrapping (Nikon 110 Eclipse ME600, Nikon Instruments spa, Italy). Meat that had never been stored in the master bag was used as a control for the display life analyses, using at least 4 trays for each display time (1 and 2 days).

4.2.3 Methods

4.2.3.1 Headspace analysis

Refer to section 3.2.3.1.

4.2.3.2 Colour evaluation and visual appearance

Refer to section 3.2.3.2.

4.2.3.3 pH

Refer to section 3.2.3.3.

4.2.3.4 Microbiological analyses

Refer to section 3.2.3.4.

4.2.3.5 Myoglobin form estimation

Refer to section 3.2.3.5.

4.2.3.6 Secondary lipid oxidation – TBA method

Refer to section 3.2.3.6.

4.2.3.7 SDS-PAGE analysis

Refer to section 3.2.3.7.

4.2.3.8 Statistical analysis

Refer to section 3.2.3.8.

4.3 Results & Discussion

4.3.1 Storage in master bags

Within 24 hours of storage, the presence of oxygen scavengers contributed to reducing the oxygen concentration inside the master bags to levels lower than the limit of detection (0.08%) of the instrumental device used. As reported by different authors (Tewari et al., 2001; Venturini et al., 2006), oxygen scavengers are an essential part of master bag solutions, providing a rapid reduction of the residual oxygen concentration and allowing the establishment of the optimal low oxygen level necessary for the reduction of myoglobin to obtain DeoxyMyoglobin (DMb). This conversion can occur at extremely low oxygen partial pressures through the reversible formation of MetMyoglobin (MMb) (Mancini & Hunt, 2005), and the rate of absorption of the gas is key to reaching the appropriate gas partial pressure in the shortest time. The anaerobic reduction of MMb is a complex mechanism that involves the surviving enzymes of the cytochrome system with nicotinamide adenine dinucleotide (NADH) as a coenzyme. MMb reduction by NADH-cytochrome reductase involves cytochrome b_5 at the mitochondrial membrane and cytochrome b at the sarcoplasmic reticulum (Bekhit & Faustman, 2005).

Although it has been established that the absence of active devices causes non-reversible discoloration, the relationship between the degree and duration of the transient discoloration of a specific cut on the one hand and the oxygen concentration of the within-package atmosphere on the other will require better definition if transient MMb formation is to be avoided (Lawrie, 1998).

Surface myoglobin determinations on the upper surface of ground beef patties were scheduled at time zero and after 4, 8 and 10 days of anoxic storage in master bags.

Storage time (days)	% OMb	% MMb	% DMb
0	94.4 (4.4) ^a	1.75 (6.3) ^a	3.85 (8.25) ^a
4	20.1 (3.3) ^b	72.9 (2.5) ^b	7 (5.1) ^a
8	36.6 (9.1) ^c	5.2 (3.2) ^{ac}	58.2 (7.0) ^b
10	58 (14) ^d	1.7 (2.5) ^{ad}	44 (12) ^c

Table 4.2. Myoglobin evolution on the upper side of ground beef patties at time 0 and after 4, 8 and 10 days of anoxic storage in master bags. Averages are reported with and standard deviations in brackets. In each column, different letters indicate a significant difference between samples stored for different times ($p < 0.05$).

At time zero, the meat was fully oxygenated on the surface; after 4 days of storage in master bag, discoloration was evident, and most myoglobin was present in the form of MMb on patties upper surface, with values of approximately $72.9 \pm 2.5\%$. Thus, a large increase in oxidized pigment after 4 days in master bags was evident, but the reduction to DMb occurred prolonging the anoxic storage, when MMb reached values lower than the initial ones. The amount of DMb did not change within the first days of storage in the master bag, but it significantly increased after the resolution of the transient discoloration; approximately 60% of the surface pigment was in the deoxygenated state at the end of the storage (Table 4.2).

Colour measurements were carried out on patties that had never been stored in the master bag and on patties stored in an anoxic environment for 4, 8 and 10 days. Chroma (C^*), which represents the saturation index, is influenced by the myoglobin content and the relative abundance of its oxidative forms, and secondary factors are the MMb concentration and total content of pigments other than myoglobin (Lindhal et al., 2001). At time 0, values of approximately 34.73 ± 1.57 were recorded (Table 4.3), and C^* decreased after 4 days in master bag to 18.14 ± 1.23 due to the oxidation of OMb. After 8 and 10 days of storage, when the transient discoloration was finally solved, C^* values increased to 28.99 ± 0.69 and 30.29 ± 1.32 , respectively. The increase in the saturation of the patty surface is directly correlated to the reduction of MMb present after 4 days of storage and its conversion to DMb.

The second colour parameter, Hue angle (H°), which indicates the overall hue of the product, is a function of the proportion of the three main forms of myoglobin (OMb, DMb and MMb) present on the meat surface. At time zero, values of approximately $19.26^\circ \pm 0.13^\circ$ were recorded (Table 4.3), matching the bright-red hue typical of fresh meat. After 4 days in anoxic storage, when the transient discoloration was still present, H° was $26.32^\circ \pm 1.87^\circ$ and subsequently decreased to lower values after 8 and 10 days of storage. At the end of the anoxic storage, the Hue angle of the patties was approximately 13° , matching the purple-red color typical of meat rich in deoxygenated pigment due to the reduction of MMb to DMb.

Storage time (days)	C^*	H°
0	$34.73 (1.57)^a$	$19.26 (0.13)^a$
4	$18.14 (1.23)^b$	$26.32 (1.87)^{ab}$
8	$28.99 (0.69)^{ac}$	$10.76 (1.20)^{ac}$
10	$30.29 (1.32)^{ac}$	$13.32 (1.51)^{ac}$

Table 4.3. Chroma and Hue angle averages (and standard deviation in brackets) of the upper side of ground beef patties at time 0 and after 4, 8 and 10 days of anoxic storage in master bags. In each column, different letters indicate a significant difference between samples.

As regarding the microbiological evaluation, during the storage in master bags, we observed a slight increase in the total viable count due to the growth of anaerobic lactic acid bacteria (Figure 4.1). The growth of the most important spoilage microorganisms of the meat, facultative *Pseudomonas* spp, was inhibited throughout the entire storage time due to the anaerobic conditions and the acidic pH. Packaging conditions might be favourable for the growth of *Brochothrix thermosphacta*, a spoilage microorganism for which meat is considered an ecological niche and that is able to produce off odours in anaerobic conditions (Ercolini et al., 2006). However, the

Brochothrix thermosphacta concentration in the patties remained constant during the whole storage period, never exceeding 10^5 CFU g^{-1} . This result may be due to the presence of LAB, which are able to inhibit the growth of several spoilage microorganisms, including *B. thermosphacta*, through competition for nutrients or the production of antimicrobial metabolites (e.g., organic acids, hydrogen peroxide, bacteriocins and reuterin) (Holzapfel et al., 1995; Vermeiren et al., 2004). Enterobacteriaceae did not grow, remaining at approximately $0.5 \cdot 10^3$ CFU g^{-1} through the end of the storage. The grinding process increases the meat surface area and the exposure of phospholipid fractions of subcellular membranes and intramuscular fat to prooxidants (e.g., iron, heme pigments) and accelerates oxidative reactions (McBride et al., 2007). Hence, it is important to follow the propagation of those reactions during the storage of meat, even if it is in anaerobic conditions, to ensure the acceptability of the products.

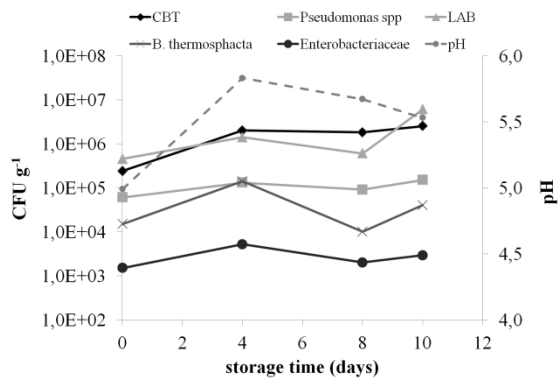


Figure 4.1. Evolution of microbiological indexes of beef patties at time 0 and after 4, 8 and 10 days of anoxic storage in master bags.

Therefore, TBARS determination (expressed as mg of malondialdehyde (MDA) per kg of meat) was performed for samples at time zero and after 4, 8 and 10 days of storage in master bags. At all analysis times, the MDA values were less than 1 mg per kg of muscle (data not shown), below the threshold of the perception of rancid flavour, which was reported to be 2 mg MDA per kg of muscle (Campo et al., 2006; Zakrys et al., 2008).

4.3.2 Blooming kinetic

The purple deoxygenated meat resulting from the anoxic storage conflicts with the most common consumer expectation, minimizing the potentiality of the system. To overcome this problem, master bags could be opened prior to displaying the meat on retail shelves, allowing the patties to undergo reoxygenation due to the high gas permeability of the wrapping material (Uboldi et al., 2013). In this case, the pigment oxygenation of ground beef has to be evaluated to understand its ability to bind oxygen after prolonged anoxic storage.

An in depth evaluation of the blooming kinetic was performed after the removing of the wrapped trays from the master bag at 4, 8 and 10 days. To evaluate the influence of air transmission through the stretch films on blooming of the upper side of the product, a manual perforation of the wrapping film was performed on half of the trays, while the remaining were maintained intact in

their wrapping film. The blooming on the upper surface of patties was followed for 300 minutes, monitoring myoglobin form evolution and colour indexes variation.

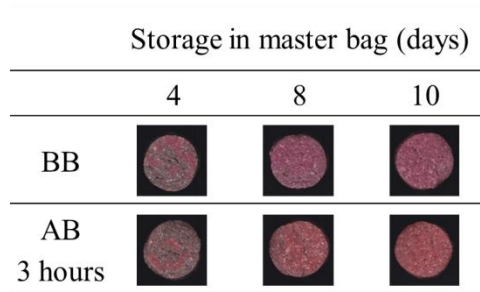


Figure 4.2. Images of patties stored 4, 8 and 10 days in master bag, before (BB) and after (AB) blooming.

The meat stored in anoxic condition for only 4 days presented a nonhomogeneous upper surface (Figure 4.2), with lighter and darker zones, due to the transient discoloration. Lighter zones had a purple-red colour typical of DMb; on the contrary, the darker colour indicates the presence of high concentration of oxidized myoglobin (MMb).

As shown in Figure 4.3a, the estimation of all myoglobin surface forms after 4 days in master bag was characterized by wide standard deviations due to the presence of non-homogeneous spots. Furthermore, in this case, the blooming could not be completed within 300 minutes, due to the persistence of spots with high percentage of irreversibly oxidized myoglobin (Figure 4.2). Also, the different oxygen transmission of the wrapping films, perforated (P) and non-perforated (UP, with an O_2TR of $22000 \text{ cm}^3 \text{ m}^{-2} \text{ 24h}^{-1}$) did not have influence on the interconversion of surface pigments. However, after the opening of the master packages, patties stored in anoxic conditions for 8 days presented a homogenous purple-red surface (Figure 4.2). The longer storage allowed the resolution of the transient discoloration through the reduction of surface pigments and the formation of DMb through the previously mentioned reducing enzyme systems in the meat (Gill, 1991; Tewari et al., 2001).

After the removal of patties from master bags at 8 days, we observed an increase of surface OMb from $34.8 \pm 14.1\%$ (before blooming) to approximately 85% with increasing blooming time (Figure 4.3b) and the simultaneous decrease of DMb on both perforated (P) and unperforated (UP) samples. The presence of a small percentage of MMb was constant during the blooming time due to the elevated presence of oxygen, which favoured myoglobin oxygenation rather than oxidation. The ANOVA showed an absence of significant differences in the level of oxygenation or oxidation of surface pigments between samples packed with P or UP PVC film, possibly because the stretch film used presented a very high O_2TR .

The results obtained after 10 days of storage inside master bags are very promising (Figure 4.3c); after the longer anoxic storage, the re-oxygenation of surface pigments occurred in a shorter time than that after 8 days. Specifically, blooming was almost complete after 60 min rather than 300 min (the blooming time necessary for maximum oxygenation after 8 days of storage in the master bag). Additionally, after 10 days of storage in the master bag, the MMb fraction on the meat surface was smaller than that of patties at the same blooming time after 8 days of storage. We hypothesize that the higher rate of myoglobin oxygenation could be favoured by the reduced competition for oxygen between pigments and respiratory activities due to the longer storage time. In fact, the slowing of physiological activities that generally consume oxygen and the inhibition of aerobic

microorganisms due to the anoxic storage may allow the gas to be available in higher quantities for pigment oxygenation (Ledward, 1992). Additionally, in this case, the kinetics of oxygenation was comparable in patties wrapped with perforated (P) or unperforated (UP) PVC film, as indicated by the lack of significant differences as assessed using ANOVA. The sole significant difference was detected when comparing the OMb percentages at all blooming times – including BB – with t0 values; only samples before blooming showed a significant difference because of the high DMb and low OMb contents. All samples that underwent at least 30 min of blooming reached an OMb content comparable to that of the time 0 samples.

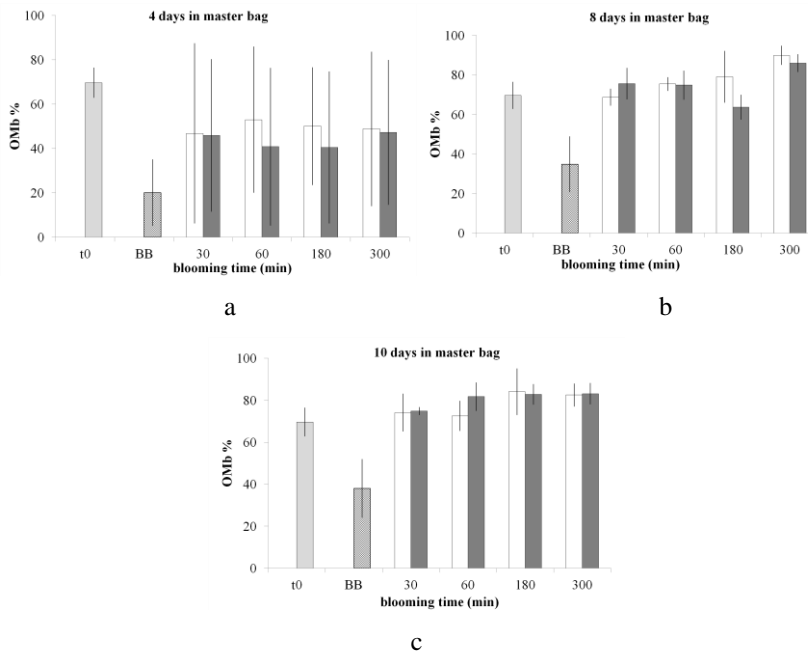


Figure 4.3. Estimation of surface myoglobin percentages at different blooming times of patties stored in the master bag for 4 (Figure 6.3a), 8 (Figure 6.3b) and 10 (Figure 6.3c) days. After the master bag was opened, half of the samples underwent perforation of the wrapping film (P, □), while the other half remained intact (UP, ■). Time zero data (□) are presented as a reference, and before blooming (BB, ▣) data were recorded prior to the perforation of the wrapping film.

The unperforated (UP) film is clearly sufficiently permeable to allow DMb oxygenation, and perforation is not necessary, especially when the product is in contact with the lid film and the headspace inside the primary package is very small, as in the experimental conditions adopted in this study.

The blooming progression on patties surface after the master bags were opened was followed by monitoring Chroma (C*) and Hue angle (H°). After 4 days of storage, both indexes registered very high standard deviations due to the presence of spots of oxidized myoglobin and spots of deoxygenated myoglobin. The transient discoloration, as previously observed for myoglobin forms, made the determination of exact values of the colorimetric indexes impossible. The results are

neither shown nor discussed given that they are not considered useful in defining the minimum blooming time for the patties.

After 8 and 10 days of storage in master bags, we observed (Table 4.4) an increase in the C* values from 0 to 30 minutes, then it stabilized and until the end of blooming (300 min), values comparable to those at time 0 were observed. After 8 and 10 days of storage in master bags, the Hue angle values were quite similar and approximately 12.6°; this value is correlated with the red portion of the colour space, in particular, the dark-red zone. H° increased after only 30 minutes of blooming and then remained constant at approximately 18°, corresponding to the bright-red colour space, for the entire period monitored (300 min).

Blooming time (min)	Hue angle (°)			Chroma		
	t0	8 days in MB	10 days in MB	t0	8 days in MB	10 days in MB
t0	19.2 (0.1) ^A			33.3 (0.3) ^A		
0		13.3 (0.8) ^{aB}	13.0 (0.4) ^{aB}		29.4 (0.6) ^{abB}	29.9 (0.7) ^{abB}
30		17.4 (0.2) ^{aC}	17.2 (0.1) ^{bcC}		31.5 (0.4) ^{aC}	33.1 (0.9) ^{baA}
60		17.5 (0.1) ^{aC}	18.0 (0.1) ^{bcC}		33.3 (0.3) ^{aA}	35.2 (1.5) ^{baA}
180		18.6 (0.1) ^{aA}	18.4 (0.1) ^{bdD}		32.8 (0.6) ^{aA}	33.8 (1.6) ^{aA}
300		18.7 (0.2) ^{aA}	18.4 (0.2) ^{bdD}		35.3 (0.5) ^{aA}	34.2 (1.0) ^{baA}

Table 4.4. Evolution of Chroma and Hue angle at different blooming times on the upper side of patties stored in the master bag for 8 (■) and 10 (□) days. ^{a-b}For each index, means in the same row with different letters differ significantly (p<0.05). ^{A-D}For each index, means of the same column differ significantly from time 0 (p<0.05).

These changes reflected the shift in the meat colour from a purple-red hue, typical of a high concentration of DMb, to a bright-red colour caused by the oxygenated pigments. As previously observed in myoglobin evaluation, the blooming phenomenon occurred faster after longer storage under anaerobic conditions due to the lower competition for available molecular oxygen between meat pigments and biological activities. Additionally, no differences (p<0.05) between samples wrapped with perforated (P) and unperforated (UP) films were detected (data not shown). In view of the above considerations, the blooming time for the ground meat patties was set at 2 hours.

4.3.3 Display life

The oxygenation obtained after blooming makes the meat ready for the shelves; thus, a display life simulation was carried out, in which meat trays were exposed in a refrigerated display case. After the master bag was opened, display life analyses were performed for 24 and 48 hours, and patties that had never been stored in the master bag were used as the control.

During the display life, the atmospheric oxygen favours the onset of oxidative reactions, especially in the case of surface proteins such as myoglobin. The oxidative state of the iron atom of this

protein is responsible of the colour and appearance of the product; therefore, we considered the MetMyoglobin increase to be a suitable quality index to follow during this step. The surface MMB percentages of patties during the display life are reported in Figure 4.4. Time 0 of the graph corresponds to the MMB concentration on the surface of patties that had never been stored in the master bag immediately after their packaging in tray-wrap solutions (lighter bar) and MMB on the surface of patties stored in the master bag for 8 days after blooming (darker bar). At this time, patties stored in the deoxygenated environment showed a significantly lower MMB concentration in comparison to the product traditionally wrapped in permeable films and never stored under anoxic conditions.

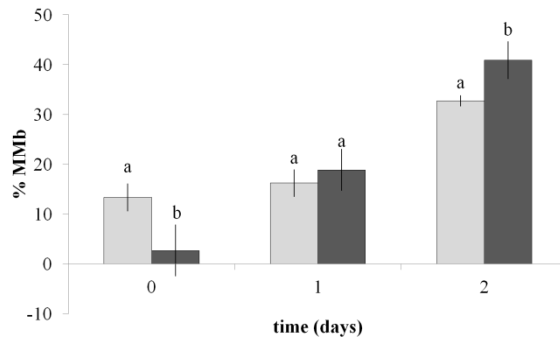


Figure 4.4. Evolution of the surface MetMyoglobin (MMb) concentration during the display life (0, 1 and 2 days) of patties never stored in the master bag (□) and patties stored in the master bag for 8 days after blooming (■). At each time, different letters indicate a significant difference between the two packaging systems ($p < 0.05$).

After 24 hours of display life, the products from both packaging systems were characterized by a comparable concentration of oxidized myoglobin, but after 48 hours, a significant difference between them was detected. Patties that had never been stored in the master bag showed a lower MMB percentage on the upper surface ($p < 0.05$). The faster oxidation of the pigments may be correlated to the longer life of the product. During storage, the antioxidant and reducing systems may remain active (Watts et al., 1966), maintaining the physiological redox state of the system, but the lack of available substrates reduces the oxygen consumption rate (OCR) of the tissue itself with increasing storage time (Tang et al., 2005). Thus, after longer storage times, the slower OCR results in higher oxygen availability for protein and lipid oxidation.

Colorimetric indexes (Table 4.5) confirmed the same variation in product appearance during the display life, moving toward hues close to brown, the typical colour of oxidized myoglobin on the meat surface. H° is considered one of the best indicators for meat discoloration (Young et al., 1999). As expected, the H° values increased during the display life, indicating the browning of the product during aerobic storage in the display case (Luciano et al., 2009; Renner, 2000).

After 48 hours of display life, the discoloration was more evident in meat stored for 8 days in the master bag.

Storage time (days)	L*	Hue angle	Chroma
0	42.8 (0.6) ^a	19.4 (0.1) ^a	36.2 (0.4) ^a
8	43.8 (0.4) ^b	18.6 (0.2) ^b	34.0 (0.4) ^b
0+1	41.9 (0.9) ^a	19.8 (0.3) ^a	30.0 (1.2) ^a
8+1	43.8 (1.1) ^b	19.3 (0.1) ^a	26.4 (0.6) ^b
0+2	42.1 (0.5) ^a	20.9 (0.1) ^a	26.8 (0.4) ^a
8+2	43.5 (0.2) ^b	23.6 (0.7) ^b	20.9 (0.1) ^b

Table 4.5. Colorimetric indexes of patties never stored in master bag (t0 = 0) and stored 8 days in master bag (t0 = 8) during the display life (+1 and +2 days). The means of 3 replicates are reported with standard deviations in brackets. ^{a-b}Different letters at each time indicate a significant difference (p<0.05).

The H° was significantly (p<0.05) higher than that of patties never stored in the master bag, most likely because the longer storage caused a wider depletion of reducing systems that were not able to balance the oxidative action of atmospheric oxygen. Additionally, although the colorimetric indexes showed a difference between samples that had never been stored in the master bag and those stored for 8 days in the master bag, no differences were visually observable, as shown in Figure 4.5.

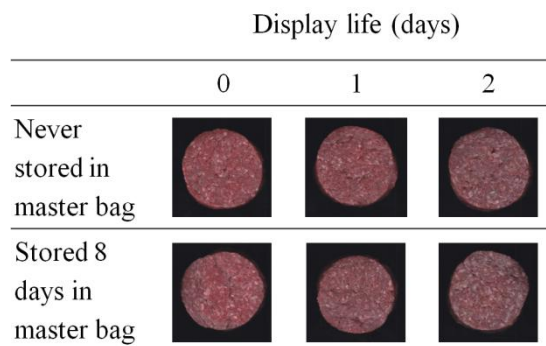


Figure 4.5. Scanning images of patties never stored in the master bag and those stored for 8 days in the master bag during the display life (+1 and +2 days).

A link between protein and lipid oxidation has been proved, which consist in the reciprocal induction of these two phenomena (Baron & Andersen, 2002; Møller & Skibsted, 2006), hence, also lipid oxidation is expected to occur during aerobic storage (i.e. display life). In order to verify this hypothesis, TBARs analysis were carried out. At t0 of display life, patties at the beginning of the test showed a lower MDA content (p<0.05) than patties stored for 8 days in the master bag (data not shown). This result may be due to the longer storage and the occurrence of protein oxidation because the formation of MetMyoglobin is highly correlated with lipid oxidation

(Chaijan, 2008). During the display life (1 and 2 days), no differences were detected, and all samples registered a MDA content of approximately 0.6 mg per kg of meat, which is much lower than the threshold of 2 mg MDA per kg of muscle.

Storage time (days)	CBT	<i>Pseudomonas</i> spp	LAB	pH
0	1.4*10 ⁵	2.0*10 ⁴	2.5*10 ⁵	5.56
0+2	3.5*10 ⁵	3.8*10 ⁴	6.0*10 ⁵	5.61
8	3.1*10 ⁶	2.0*10 ⁴	4.1*10 ⁶	5.58
8+2	2.8*10 ⁶	7.0*10 ³	6.3*10 ⁶	5.56

Table 4.6. Evolution of microbiological indexes (CFU g⁻¹) and pH of beef patties after 0 and 2 days of display life (+2), after 0 and 8 days of storage in the master bag.

Microbiological analyses and pH measurements were also performed during the display life of the control samples and patties stored for 8 days in the master bag (Table 4.6). It is evident that during the 2 days of exposition in the display case, microorganisms exhibited no growth in patties removed from the master bag, most likely due to the residual antibacterial effect of the CO₂ present in the master bag itself. In contrast, patties never stored in the master bag showed an increase in all indexes during the display life. It is important to highlight that the microbial content (especially CBT and LAB) in patties from the master bags was higher at the beginning of the display life compared to time 0 of control samples due to the longer storage leading to an increase in CBT and LAB of almost one order of magnitude.

4.4 Conclusions

The optimization of a master bag packaging system for ground beef patties was carried out successfully during this work. A suitable storage time in the master bag (anoxic conditions) was identified as 8 or 10 days. After both of these periods, the blooming phase (i.e., reoxygenation of surface pigments) was optimized, and the blooming time was set at 2 hours. Additionally, the perforation of the stretch film wrapping the product was carried out on some samples to evaluate its contribution in favouring blooming, but the effect of this procedure was statistically ($p < 0.05$) non-significant.

It is possible to conclude that the investigated master bag packaging system is able to prolong the shelf life of ground beef patties, ensuring a storage time at least of 10 days and a 2-days display life. Hence, the master bag system could replace the traditional one, facilitating the management of a very perishable product and, at the same time, ensuring its quality.

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5. Low oxygen partial pressures: Sliced beef meat chemical and biochemical quality evolution.

5.1 Introduction

Meat that is prepared at retail level is usually part of boxed meat systems where primal cuts are produced and often vacuum packaged (VP) at the packing plant and distributed to the retail centres. There, the primal cuts are further processed (cut, minced, etc.) with the aim to package in consumer units that afterwards are stored in refrigerated display for few days. The most important trend that stands out year after year in case-ready meat packaging is the need to reduce labour in the back of the retail store (Belcher, 2006).

One solution consists in packaging the meat (already prepared as the final cut) in trays overwrapped with a high gas permeability film, then enclosed in a larger master bag film that contains multiple packages. When a retailer needs product to stock the shelf, he opens the master bag and removes the packages from the bag. The product should bloom forming the desirable, bright, cherry-red colour as function of the oxygen permeability of the film that overwraps individual trays. The absence of O₂ in low-oxygen packages usually maintains myoglobin in a reversibly reduced state (thus having a purplish-red colour, unfamiliar to many consumers) and minimizes oxidative deteriorative reactions. The limit of this kind of packaging is that a very low oxygen concentration is required to maintain myoglobin in a deoxygenated state and stringent optimization of the whole packaging system is also needed. Different information about the O₂ critical levels for meat is available in the literature. Gill (1996) underlined that an O₂ residual level at a maximum of 0.1%, but preferentially, at no more than 0.05% is recommended to guarantee the blooming of meat upon re-exposure to air. Similar results were reported by Mancini and Hunt (2005), who indicated a critical value of 0.05% O₂ (500 ppm) for beef. Tewari et al. (2001) distinguished a limit lower than 100 ppm for beef with poor colour stability and lower than 600 ppm for beef with high colour stability when stored at sub-zero temperatures. Again, for ground beef, oxygen levels lower than 10 ppm were found to optimize red colour stability of beef during retail display (Gill & McGinnis, 1995). In fact, a critical point is that, at low O₂ concentration, the oxidation of DeoxyMyoglobin to MetMyoglobin is faster than the oxidation of OxyMyoglobin (Venturini, 2006), with a higher risk in colour changes during the distribution life.

To control these changes, CO at low levels has been permitted in some Countries as MAP gas for use during distribution (Cornforth & Hunt, 2008) and its application to low-oxygen packaging of fresh meat in master bags is a consolidated reality (Jeong & Claus, 2010). In this way, during storage, the bright, cherry-red colour of meat is assured because CO binds strongly to myoglobin to form the extremely stable CarboxyMyoglobin that also reduces the oxygen consumption rate of the muscles (Seyfert et al, 2007).

However, the adoption of this packaging technology is strongly limited in centralized plants where CO is not permitted, especially for the difficulty in managing the colour changes that can occur during the storage (i.e. the transient discoloration) and during blooming. To enable the exposition of the beef meat on the display in the red form, the anoxic packaging unit has to be correctly dimensioned and optimized, controlling, first of all, the oxygen concentration after the master bag sealing and during the storage.

Until now, different studies have addressed the efficacy of oxygen-depleted atmospheres in conjunction with CO₂ to prevent transient and permanent discoloration of different beef cuts, focusing the attention on the changes during the display life (Isdell et al. 1999; Tewari et al., 2002a; Venturini et al., 2006) but no conclusive results were obtained, especially concerning the quality

attributes change over the anoxic storage and the re-oxygenation step. From these works it is evident that the success of this kind of packaging system depends on different factors, both intrinsic (i.e. age of animal, muscle type, post mortem age, pre-treatments etc.) and extrinsic (i.e. temperature, oxygen availability, packaging materials, headspace volume etc.); therefore it is very difficult to define the optimum conditions.

The potentiality of an oxygen-depleted master bag system in prolonging the distribution life needs to be further investigated, to better understand the changes in quality and safety attributes, especially if associated to the residual oxygen partial pressure. For this reason, the present study analyses the effects of oxygen-depleted atmospheres in maintaining the quality of beef steaks from *Semiteminosus* muscle in case-ready units stored inside a master bag where oxygen scavengers are used with CO₂ and N₂ as modified atmosphere.

Three main steps of the storage are described and discussed. First the evolution of beef meat quality was studied during the storage in master bag at about 0-1°C. The main changes that happen during the blooming at 4°C, once the master bag is open, were addressed next. Finally, modifications of the case-ready meat were studied after the trays were exposed on the shelves at 4-5°C. Not only chemical, microbiological and visual indexes, but also biochemical evaluation (e.g. iron oxidative state, reduced glutathione), were considered to describe the quality changes and to understand the effects of this packaging system for *Semiteminosus* muscle.

The results obtained in this study are presented considering the three main steps of the packaging system: storage of the meat in the master bag units and blooming followed by the display life in air.

5. 2 Materials & Methods

5.2.1 Meat cuts

Sliced beef (Italian breed Piemontese) from *Semiteminosus* muscle, 1 cm thickness were tested. The primal cut was never stored under vacuum.

5.2.2 Packaging system

Piemontese beef slices were packed into non barrier EPS trays (180*250*30 mm) and wrapped with a high permeability stretch film PVC-based, with a OTR of 22000 cm³ m⁻² 24h⁻¹ bar⁻¹ (23°C and 0% RH). Two slices were put in each tray, for a total weight of about 300-400 g, and the wrapping film was manually perforated. Two trays were inserted in a barrier master bag (PE/EVOH/PE, 58*43cm, O₂TR < 0.01 cm³m⁻² 24h⁻¹bar⁻¹ at 23°C) with two oxygen scavengers (FreshPax[®] CR 8, Multisorb Technologies). The master bag were sealed in modified atmosphere (30% CO₂ and 70% N₂) using a CVP machine (Downers Grove, IL), applying a double vacuum-flush cycle at the following conditions: vacuum 7 s, flushing 0.8 s for the first cycle; vacuum 6.5 s, flushing 3 s for the second cycle. The sealing time was 2.7 s. Meat was stored in the dark for 21 days at 0.5±0.5°C: master bag were opened after 4, 7, 14 and 21 days, then meat was allowed to reoxygenate (blooming) at 4±1°C and finally exposed onto a display case for 48 hours in order to simulate the display life at retail level. At each opening time, meat was analysed before (BB) and after (AB) blooming, and after 24 and 48 hours of display life.

5.2.3 Methods

5.2.3.1 Headspace analysis

Residual oxygen concentration inside master bag was assessed as described in section 3.2.3.1.

Residual oxygen concentration inside the trays was assessed by gas chromatography using a GC (HP 5890 series 2) fitted with a TCD detector. The inlet temperature was set at 100°C. The GC was equipped with a steel packed column (2 m 6 mm, CTR I Alltech, Milano), which was held isothermally at 50°C. The carrier gas used was He, and the pressure was set at 140kPa. The run time was 6 minutes and the chromatogram was recorded and elaborated using Azur (Kromatek, UK). 30µl of headspace were sampled and then injected in the GC using a gastight syringe. Analyses were carried out at specific times for each test.

5.2.3.2 Colour evaluation and visual appearance

Refer to section 3.2.3.2.

5.2.3.3 pH

Refer to section 3.2.3.3.

5.2.3.4 Microbiological analyses

Refer to section 3.2.3.4.

5.2.3.5 Myoglobin form estimation

Refer to section 3.2.3.5.

5.2.3.6 Determination of myoglobin heme-iron oxidative status: Electronic Paramagnetic Resonance

Samples for EPR analysis were prepared after 0, 0.25, 1, 1.25, 2, 2.25, 3, 3.25, 4 and 7 days of storage in master bag. Master bag opening, meat insertion into EPR tubes and freezing of samples in liquid nitrogen were carried out inside an anaerobic chamber (855-AC, Plas Labs, USA), filled with an anoxic gas mixture containing 5% hydrogen, necessary for the oxygen scavenging activity of the palladium-based device. Samples were kept at -80°C until analysed.

EPR spectra were acquired using a Bruker ESP 300 X band spectrometer, equipped with the ESP 1600 data acquisition system. The experimental conditions were as follows: 9.28 GHz radiation, 1100±300 G magnetic field, temperature 77 K. For the analysis, EPR tubes containing the sample were inserted into a finger dewar containing liquid nitrogen, inserted into the TE₁₀₂ (ER4201, Bruker) standard rectangular cavity.

The EPR spectrometer irradiates the sample with a constant electromagnetic radiation and at the same time varies a magnetic field (microwaves) to achieve electron resonance of the molecule of interest. The spectrometer detects the absorption of the electromagnetic radiation by the targeted compound as function of the magnetic field applied, and record it as a first derivative spectrum.

The absorption of the radiation is observed when the energy of the irradiation matches the energy difference between the two spin states of the targeted molecule. The structure and intensity of the signal depends on the targeted compound nature and concentration. The EPR measurement were performed by prof. Rita Guzzi at Physical Department, University of Calabria, Rende (Cosenza).

5.2.3.7 Secondary lipid oxidation – TBA method

Refer to section 3.2.3.6.

5.2.3.8 SDS-PAGE analysis

Refer to section 3.2.3.7.

5.2.3.9 Determination of reduced glutathione (GSH) trough DTNB assay

The method used is based on the reaction of glutathione SH group with a specific reagent, DTNB (5,5'-dithiobis-(2-nitrobenzoic acid), Ellman's reagent). 2g of meat were homogenized in 10ml of phosphate buffer (50mM, pH 6.8) using a manual Potter, then the homogenate was centrifuged for 20 min at 8000 x g, then 20 min at 12000 x g using a Beckman centrifuge (J2-21M/E, Beckman, USA). The supernatant was acidified with sulfosalicylic acid (5% w/v final), in order to prevent the oxidation of free SH groups due to oxidative enzymes and to the low pH. The acidified extract was then centrifuged using a microcentrifuge (Mikro 20, Hettich Zentrifugen, UK). Before the DTNB addition, the extract was neutralised neutralized adding solid TRIS until neutral pH was reached. A proper amount of neutralised solution (100 μ l) was added to 900 μ l of DNTB solution (0.2 M in phosphate buffer 50mM, pH 7): DTNB added to the sample formed a mixed disulfide with thiolic molecules, such as GSH, with the consequent formation of a yellow dianion, which absorbance is detected at 412nm. The acquisition of absorbance values was performed using a spectrophotometr (Lambda 2, Perkin Elmer, Italy). The DTNB solution was used as blank, since the contribution of myoglobin to absorbance at 412 nm in diluted meat homogenates is reported to be negligible (Hofmann & Reiner, 1978). The GSH content was calculated using a calibration curve obtained with GSH standard solutions.

5.2.3.10 Statistical analysis

During the storage in master bag, the influence of different packaging system (with and without scavengers) on meat quality indexes was compared statistically for significant differences ($p < 0.05$) using one way analysis of variance (Software Statgraphics Plus for Windows, v.4). After blooming, one way analysis of variance was carried out to assess the effect of the storage time in master bag (7 and 21 d) and in the traditional packaging solution (tray-wrap solution) on quality indexes. Duncan's test was used to determine significant differences between individual means when the treatment effect was significant ($p < 0.05$).

5.3 Results

5.3.1 Storage in the master bag

The success of this kind of packaging solution for fresh beef is based on the oxygen concentration that -remains inside the master bags and that is available to the meat during the long storage. At

least, four main factors can influence this residue: *i)* the effectiveness of the MAP equipment in removing air; *ii)* the permeability of the tray-wrap packaging materials in enabling the oxygen exchanges from the headspace of the tray towards the master bag headspace; *iii)* the oxygen scavengers absorbing kinetic *iiii)* the oxygen uptake of the muscle itself. In fact, two isolated systems affect the O₂ concentration in the overall package-atmosphere of master bags (Tewari et al., 2001), namely the headspace of the master bag and the headspace of the tray inserted into the master bag itself (i.e. the primary packaging). The air initially present in a bag is usually evacuated for a set time that will inevitably leave substantial residual volumes of oxygen within packs (Gill & Gill, 2009). For this reason, the MAP equipment has to be set in such a way that the residual volume of oxygen in the master bag is minimized. Also the oxygen permeability of the primary packaging (tray and wrapping film) plays an important role: the higher the permeability, the higher the diffusion of the air towards the master bag headspace during evacuation. Moreover, oxygen entrapped in the tray headspace or inside the tray structure itself (a foam, in the case of our experiment) should rapidly permeate in order to be scavenged before the consumption of oxygen by meat itself. The complexity of these exchanges requires that the oxygen scavenger is efficacious enough, in terms of absorbing capacity and absorption rate, at low temperatures and at low oxygen concentrations, before meat could act itself as a scavenger.

Time (days)	Without O ₂ scavenger		With O ₂ scavenger	
	%	O ₂ partial pressure (mbar)	%	O ₂ Pressure (mbar)
0.0	1.08 (0.27)	10.96 (3.22)	1.13 (0.22)	11.46 (2.32)
0.6	1.15 (0.32)	11.65 (2.21)	0.87 (0.35)	8.82 (3.55)
1.0	1.49 (0.42)	15.05 (4.18)	< LOD	< LOD
1.5	1.82 (0.20)	18.37 (2.04)	< LOD	< LOD
2.5	1.67 (0.15)	16.92 (1.52)	< LOD	< LOD
4.0	1.96 (0.46)	19.85 (4.59)	< LOD	< LOD
7.0	1.95 (0.31)	22.00 (3.12)	< LOD	< LOD
10.0	1.25 (0.02)	12.82 (0.20)	< LOD	< LOD
14.0	1.10 (0.03)	11.22 (0.30)	< LOD	< LOD
21.0	1.00 (0.46)	10.23 (4.69)	< LOD	< LOD

Table 5.1. Oxygen concentration and partial pressures in the master bags with and without O₂ scavengers (standard deviations into brackets).

In this work, the oxygen concentration during storage was measured through a non-invasive device and the results are shown in Table 5.1. The mean residual of O₂ estimated inside the master bags immediately after their closure, was 1.1±0.3%, corresponding to about 10.96 mbar. This value is not compatible with the fresh meat storage. In fact, McMillin (2008) reported that fresh beef meat is very susceptible to MetMyoglobin formation at low O₂ pressure (in the 6-13.3 mbar range).

Although at low oxygen concentrations the gas availability limits the capacity to absorb oxygen (as well documented by Brandon et al., 2009 and by Tewari et al., 2002b), the scavengers used in this study allowed the absorption of residual O₂ inside the master bags; in fact, during the first 24 hours the concentration dropped up to values not detectable by the instrument (i.e. lower than 0.08%), creating an oxygen-depleted environment. In the master bags without oxygen scavengers, oxygen raised up to about 2% during the first 48 h and then progressively decreased, remaining constant at values higher than 1%. Previous works that describe the use of oxygen scavengers in low-oxygen modified atmosphere packaging showed that the presence of the active device inside the master bags can really maintain the quality of meat. However, results concerning the number of the scavengers, their position and the real effectiveness in absorbing oxygen are not always concordant. The experimental work described by Venturini et al. (2006) showed that concentration of oxygen lower than the critical level for the formation of MetMyoglobin was reached only after 14 days of storage. As well underlined by the Authors, the oxygen scavenger system used was underestimated (in terms of number and effectiveness) and/or the expanded polystyrene trays affected the concentration of the O₂ in the master bags.

In their work, Tewari et al. (2002a) reported on the need of using multiple O₂ scavengers (eight or more) to achieve an oxygen concentration of ≤ 500 ppm in the pack atmosphere and for a master bag of a specific size (595 x 447 mm). In the same work, the Authors suggested that placing O₂ scavengers inside the retail trays might have reduced the number of O₂ scavengers. Isdell et al. (1999) reported O₂ concentrations of 500 ppm after storing master packaged steaks at 0°C for several weeks and placing only one O₂ scavenger in each retail tray. However, in all these studies, no information about the effect of exudates in contact with the scavengers was evidenced.

The half reduction of oxygen concentration is due to the uptake by the muscle that plays an important role in the colour changes during storage and depends strongly from the muscle typology (Tang et al., 2005): in general, *Semitendinosus* muscle has a low oxygen consumption rate, if compared to other muscles, because of the greater α -white fibre content that are characterized by less mitochondria (Seyfert et al., 2007).

Time (days)	With O ₂ scavenger			Without O ₂ scavenger		
	MBC	<i>Pseudomonas</i> spp.	LAB	MBC	<i>Pseudomonas</i> spp.	LAB
0	3.4	3.0	2.0	3.3	3.1	2.2
7	4.4	3.3	5.5	6.8	5.0	6.4
11	5.0	3.5	5.8	-	-	-
14	5.0	3.0	5.3	-	-	-
21	5.1	3.2	5.7	-	-	-

Table 5.2. Microbial evolution during the storage inside master bags (data expressed as log CFU g⁻¹)

As mentioned by Nychas et al. (2008) a vast number of studies in meat microbiology have established that spoilage is caused only by the fraction of the initial microbial association that dominates. Before packaging, the mesophilic bacterial count was prevalently dominated by *Pseudomonas* spp. that is in most cases responsible for spoilage of meat stored aerobically.

Actually, in master bags without scavengers the residual oxygen (higher than 1%) promoted the development of the facultative *Pseudomonas* population, that passed from 3 to 5 log CFU g⁻¹ during the first week of storage without reaching the limit values (around log 7-8 CFU g⁻¹) associated to slime and off-odours formation (Nychas et al., 2008) (Table 5.2). At the same time, the combination of low oxygen concentrations with the presence of carbon dioxide promoted the growth of LAB that increased from 2 to 6.4 log CFU g⁻¹ after 7 days of storage.

In meat stored in master bags with O₂ scavengers, lactic acid bacteria count increased from 2 to 5.7 CFU g⁻¹, while the number of *Pseudomonas* spp. remained lower than 3.5 CFU g⁻¹ (Table 5.2). It is assumed that the growth of *Pseudomonas* spp. was inhibited by the absence of oxygen and/or high carbon dioxide concentrations created in the master bags and also by the acid and antimicrobial substances produced by lactic acid bacteria (Lee & Yoon, 2001). In fact, the mean pH value of the samples before packaging was 5.75 ± 0.03 and it decreased up to 5.50 ± 0.05 at the end of the three weeks of storage into the master bag. These results are in line with the study of Sakala and others (2002) in which the decrease in pH value by around 0.25 units during the same interval of storage was observed in vacuum-packaged fresh beef. The decrease in the pH of the meat may therefore be attributed to the predominance in lactic acid bacteria.

In both packaging solutions, Enterobacteriaceae remained always lower than 1 CFU g⁻¹; in the same way, *Brochotrix thermosphacta*, *Escherichia coli* and spores of *Clostridium perfringens* were always absent, in all tested samples.

After slaughtering, a tenderizing process starts in the muscle, thanks to the activity of proteolytic enzymes, in particular calpains and proteasomes (mainly cathepsin) (Ouali et al., 2006). This process is influenced by many factors, including the rate of depletion of the available energy and tissue acidification, due to the onset of anaerobic metabolism (Huff Lonergan et al., 2010). At the same time, programmed cell death occurs. This phenomenon is called *apoptosis*, and represents a natural mechanism that eliminates excessive, damaged or potentially dangerous cells (Herrera-Mendez et al., 2006). Extreme conditions established into the master bags during anoxic storage could promote all these events and accelerate proteolysis. However, no differences were evident between the protein pattern of samples never stored in a master bag and samples stored in a master bag for 7, 14 and 21 days (data not shown).

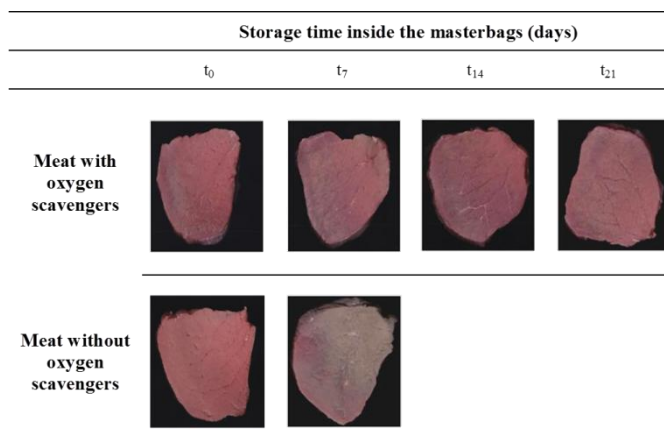


Figure 5.1. Digitalized images of the meat slices stored in master bag with and without oxygen scavengers

Figure 5.1 shows the full colour digital images of meat slices, making it evident that storage without oxygen scavengers induced formation of MetMb on the surface of the meat, thus invalidating acceptance by the consumer. As reported by Mancini and Hunt (2005), there is a strong association between colour preference and purchasing intent with consumers discriminating against beef that is not red (i.e. beef that is purple or brown). Therefore, visual determinations are the gold standard for assessing treatment effects and estimating consumer perception. For this reason, the storage without scavengers was stopped after 7 days, without performing display life simulation.

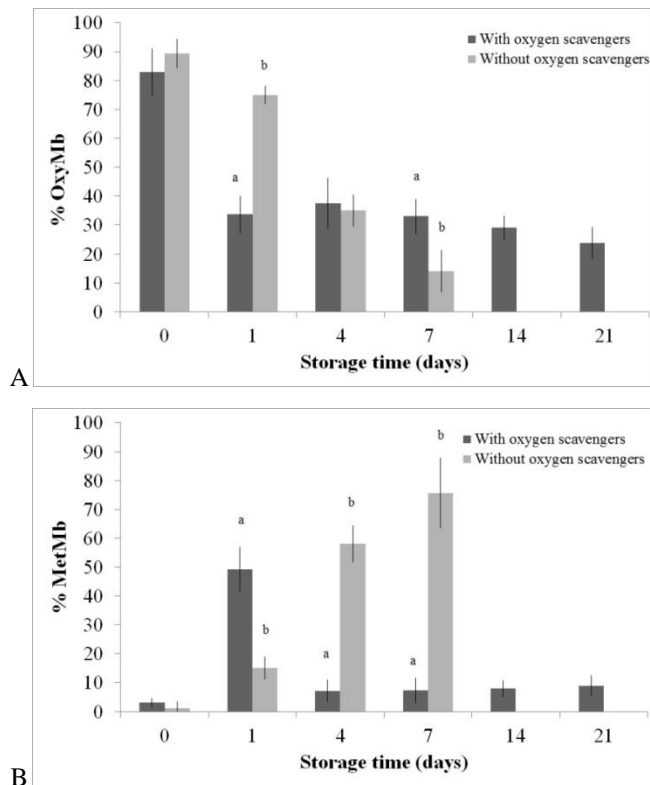


Figure 5.2. Evolution of myoglobin form levels on the beef surface during the storage in the master bag with and without scavengers: A) OxyMb; B) MetMb. ^{a-b} storage treatments are significantly different ($p < 0.05$) if they have no common superscript letter.

In this work, the relative percentage of OxyMyoglobin (OxyMb), DeoxyMyoglobin (DeoxyMb) and MetMyoglobin (MetMb) on the surface of muscle were estimated using the methodology and the formulas specified by AMSA, as described in the Materials and Methods section.

Figure 5.2A shows the time course of OxyMb disappearance during the storage in the master bags. Progressive loss of OxyMb form is slower in the absence of scavengers than in their presence, although in the latter case about 30% OxyMb remains after the first week of storage. This is explainable taking into consideration the typical hyperbolic shape of the oxygen dissociation curve for beef myoglobin characterized by a very low P50 (i.e. the point at where myoglobin is exactly

half saturated with oxygen) equal to about 2 mbar. In our system, after 1 day of storage in master bag with scavengers, oxygen residual is lower than the detection limit of the gas analyzer (0.08%, which corresponds to about 0.8 mbar). At this partial pressure, the % oxygen saturation is still 20-30%, justifying the OxyMb values found during the storage in the master bag.

The oxygen decrease inside the master bags promoted the formation of DeoxyMb form that was by far more pronounced in the presence of scavengers, reaching average values of 50% (data not shown). In meat stored without scavengers, the evolution of the myoglobin forms was quite different. In fact, the OxyMb decreased slowly during the first week of storage (Figure 5.2A) reaching values of about 14% and low concentrations of DeoxyMb were estimated (data not shown). At the seventh day of storage, MetMb reached values around 75% (Figure 5.2B) and the discoloration was permanent, as also evidenced in Figure 5.1.

Steaks stored in master bags with oxygen scavengers underwent transient discoloration. In fact, the MetMb content increased during the first days of storage and then decreased after 7 days of storage, remaining at constant and very low values (Figure 5.2B). This phenomenon has been described by other authors (O’Keeffe & Hood, 1980-81 a,b; Tewari et al., 2001; Mancini & Hunt, 2005). In particular conditions, OxyMb is not converted directly to DeoxyMb, but first undergoes reversible oxidation to a ferric redox state (Figure 5.3).

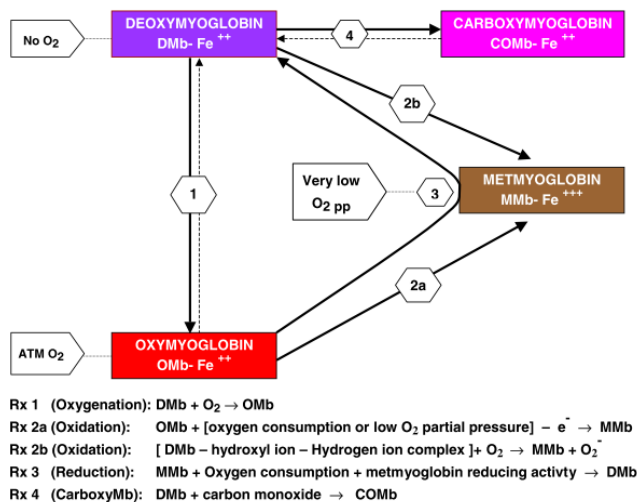


Figure 5.3 Visible myoglobin redox interconversion on meat surface. From: Mancini & Hunt, 2005.

Oxygen-utilizing enzymes yet present in *Semitendinosus* muscle 13 days post mortem may be involved in depleting oxygen at or near meat surface and favouring formation of MetMb. Other enzymatic reducing systems are then involved in converting MetMb to ferrous Mb (Bekhit & Faustman, 2005). The MetMyoglobin reducing activity (MRA) of muscle tissue is limited, and once exhausted cannot convert back to the ferrous form any of the MetMb formed. Mancini and Hunt (2005) hypothesized that the coupled reactions of myoglobin oxidation and reduction may occur only at very low (but non-zero) oxygen partial pressure, assuming that the reduction of MetMb under totally anoxic conditions was unlikely. In other words, if no oxygen is available, the tissue cannot couple oxygen consumption to the reduction of ferric to ferrous iron. However, the

oxygen concentration cannot be higher than the limit over which the irreversible oxidation of OxyMb to MetMb occurs. This is evident looking at the results: within 1 day of storage, the oxygen concentration is lower than 1% inside the master bag headspace (further decreased to less than the LOD of 0.08% after 1 day) and the reversible formation of MetMb suggested that its reduction occurred at very low oxygen concentrations. In meat stored without scavengers, the evolution of the myoglobin forms was quite different: in fact, the OxyMb decreased slowly during the first week of storage and low concentrations of DeoxyMb were estimated. Progressively, the MetMb concentration increased reaching values of about 75% and the discoloration was no transient but permanent. For this reason, as also supported by the colour and visual appearance of the product that are discussed later, the storage of the meat without the oxygen scavenger was stopped after one week.

In general, transient discoloration of meat is not a major concern when the product is in storage, transit, or both for periods longer than 2 days (Gill & McGinnis, 1995). Such discoloration can be problematic only when commercial conditions require rapid distribution and display of the product. The residual oxygen inside the package plays an important role in the transient discoloration of the meat but probably other factors could be involved and for this reason other investigation is required. In order to identify the role of redox state of iron in the phenomena described above, we used EPR technique that is specific for defining the redox state of iron in various matrixes.

The EPR analyses take advantage of the fact that require a minimal manipulation of the sample prior to analysis, thus a reduction of external stress which may cause significant changes in the oxidative state and morphology of proteins. In fact, the only step required is the insertion of a small portion of meat inside EPR quartz tubes. Moreover, the possibility to prepare samples inside an anaerobic chamber, helped in preventing the occurrence of any reaction possibly initiated by atmospheric oxygen, thus minimizing changes in the chemical properties of the sample that was kept in liquid nitrogen until analysed. This is the major difference in respect to spectrophotometric determination; in fact, in the case of absorbance spectra acquisition, the sample is exposed to air, for a minimum period of time, between the opening of the package and the spectra recording.

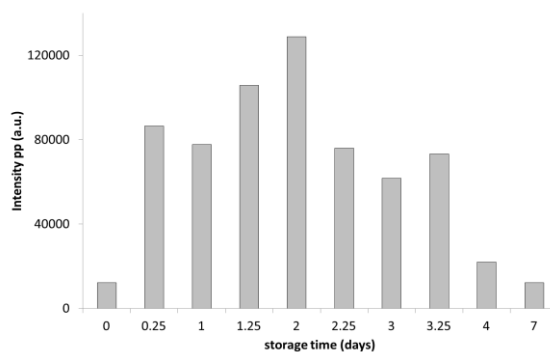


Figure 5.4. Peak intensity of the myoglobin heme iron signal of beef meat slices stored in master bag for 7 days.

So far, the use of EPR has been applied only on model systems, generally solutions of heme proteins, for different aims, such as the evaluation of myoglobin effectiveness as photosensitizers in singlet oxygen generation (Whang & Peng, 1988), and the detection of radicals derived from nitrite-mediated myoglobin oxidation (Keszler et al., 2006). The use of EPR for the detection of

heme iron oxidation is rarely reported, as in the case of Bhattacharjee and Chakraborti (2011), which again worked on a model system.

Myoglobin is a water-soluble globular protein which mainly serve as a temporary store of oxygen, thanks to the presence of an heme ring (i.e. a protoporphyrin ring surrounding an iron atom). The heme iron binds four nitrogen atoms of the protoporphyrin and an histidine side-chain of myoglobin which secures the heme in the hydrophobic pocket. The sixth ligand is available for binding small molecules, such as oxygen, carbon monoxide and nitric oxide, depending on the oxidative state of iron. The α -eliches of myoglobin tertiary structure are folded in order to sterically protect heme from oxidation, but at the same time they create some fluctuating pathways to allow oxygen bound and release (Cohen et al., 2006). Thus, the oxygen concentration surrounding the molecule is involved in the sterical organization of the molecule as well as the oxidative status of the heme iron.

In Figure 5.4 the intensity of the EPR signal of myoglobin heme iron of fresh meat samples stored in master bag for 7 days is reported. The intensity of the peak is directly correlated to the degree of iron oxidation: the higher intensity is obtained after only 1 day of storage, when a great MetMyoglobin concentration is present; while, after 4 days in anoxic environment, the intensity of the signal decreases to level comparable to the initial one. At this time the transient discoloration has been solved, since heme iron is in its reduced state.

Time (days)	Hue angle (°)			Chroma		
	With O ₂ scavenger	Without O ₂ scavenger	S*	With O ₂ scavenger	Without O ₂ scavenger	S*
0	19.1 (3.2)	18.7 (0.5)	NS	34.8 (5.6)	36.2 (1.5)	NS
1	20.9 ^a (3.3)	27.5 ^b (1.7)	p<0.001	21.3 ^a (1.7)	16.0 ^b (2.2)	p<0.001
4	14.3 ^a (2.0)	34.4 ^b (3.4)	p<0.001	27.2 ^a (0.9)	18.0 ^b (3.1)	p<0.001
7	15.2 ^a (1.7)	40.3 ^b (1.1)	p<0.001	27.2 ^a (1.6)	16.4 ^b (2.7)	p<0.001
14	15.6 (2.1)	-	-	25.8 (1.9)	-	-
21	16.2 (2.9)	-	-	28.0 (3.1)	-	-

*S=significance

^{a-b} Packaging systems are different (p<0.05) if they have no common superscript letters

Table 5.3. Evolution of Hue angle and Chroma indexes during the storage in master bag, with and without scavengers

^{a-b} Means of the same row (for each colour index) with different letters differ significantly (p<0.05).

^{A-D} Means of the same column with different letters differ significantly (p<0.05)

The evolution of the colour indexes is summarized in Table 5.3. A hue angle equal to 0 indicates a dark red colour, and as hue angle increases to 90 degrees, redness decreases and yellowness increases. As evident in Table 5.3, comparing values in each column, hue angle increased within 1 day of storage into master bags indicating an increase in MetMb form (Lindahl et al., 2001). As demonstrated previously, this represents a transient and reversible event. In fact, after that time, the value declined maintaining constant over the storage period. Also the Chroma index had a double

trend: within the 24 hours of storage the value dropped and then increased and maintained a lower level than the fresh product, indicating a less vivid colour.

As shown in Table 5.3, at each storage time, both Chroma and Hue angle values were significantly different between samples with and without scavenger (comparison between the rows of each colour index). Meat stored without the active device underwent a strong decrease in Chroma values caused by the MetMyoglobin formation that reached the maximum value after 7 days of storage, as also suggested by the data discussed before. Also the Hue angle index changed, increasing immediately after storage up to very high values (40.3 ± 1.1).

Lipid oxidation is one of the main factors limiting the quality and acceptability of meats and other muscle foods (Chaijan, 2008). In this study, TBARS determination was performed since this parameter is one of the most widely used tests for evaluating the extension of secondary oxidation.

Green and Cumuze (1981) found that oxidized flavour in beef was detected over a broad range of TBARS from 0.6 to 2.0 (expressed as mg malondialdehyde-MDA per kg of meat) indicating a large variation in the threshold of the panelists. More recently, Campo et al. (2006) and Zakrys et al. (2008) reported a value of about 2 mg MDA per kg of muscle as the limiting threshold for acceptability of oxidation in beef because it could be considered the limiting point from where rancid flavour overpowers beef flavour, and therefore, to be considered as the maximum level for positive sensory perception of beef.

Secondary lipid oxidation, expressed as mg MDA per kg of muscle in meat stored in master bags with and without oxygen scavengers, never exceeded the threshold values indicated above, regardless of the presence/absence of oxygen scavengers. However, in the absence of scavengers, the MDA content increased markedly during the first seven days of storage, passing from 0.032 to 0.270 mg MDA kg⁻¹ muscle. On the contrary, in the presence of scavengers, the level of MDA increased only slightly over the entire storage period. Our data support the general observation that lipid oxidation in meat is proportional to the concentration of oxygen present and is expected to be minimal in low pO₂ environments.

Moreover, it is well known that lipid and myoglobin oxidation in meat are strictly correlated, and generate products that can further accelerate oxidation in a reciprocal manner (Baron & Andersen, 2002; Moller & Skibsted, 2006; Chaijan, 2008). As well described by Faustman et al. (2010) and Moller and Skibsted (2006), the oxidation of OxyMb to MetMb generates reactive intermediates (like the superoxide anion radical and hydrogen peroxide) capable of enhancing further oxidation of OxyMb and/or unsaturated fatty acids. In particular, the myoglobin in its ferric state (MetMb) has been found to have a pseudo-peroxidase activity which forms several pro-oxidative Mb species during the catalytic cycle, following reaction with H₂O₂ and other peroxides. MetMb also takes part in the propagation of lipid peroxidation by cleavage of lipid hydroperoxides. In addition, H₂O₂ may also be produced in a significant quantity as a result of growth of the catalase-negative lactic acid bacteria on the meat surface (Holzapfel et al., 1995).

However, a lack of a clear tie between these oxidative reactions exists at very low (but non-zero) oxygen concentrations (Faustman et al., 2010). Our data suggest that two different pathways are involved in the oxidative outcomes observed in meat during the first days of storage in master bags with and without oxygen scavengers, and that oxygen availability is the most probable discriminator. At oxygen partial pressures lower than 10 mbar (Table 5.1), meat undergoes transient changes in myoglobin redox state (formation of MetMb and afterwards of DeoxyMb *via* MRA systems), while the lipid oxidation does not change in a significant way. On the contrary, oxygen partial pressures higher than 10 mbar promote at the same time the irreversible oxidation of OxyMb and a more evident secondary lipid oxidation. As also suggested by Faustman et al. (2010),

in this frame it would be of high interest to understand the competition of the oxygen consumption by all possible candidates (i.e. mitochondrial activity, bacterial metabolism, lipid oxidation etc.).

5.3.2 Blooming

Most red meat is marketed in its OxyMb form, and the myoglobin of meat stored in low oxygen systems must oxygenate or bloom prior to display (Beggan et al., 2006) to promote OxyMb formation. Blooming is the result of oxygen bound to the iron atom: in this state the myoglobin molecule is called OxyMyoglobin.

After 7, 14 and 21 days of storage, the wrapped trays were removed from the master bags and stored at about 3°C in the dark. The blooming time was fixed at 4 hours but it progressively decreased to 30 minutes with the increase of the storage of meat in the master bags.

Figure 5.5 shows the OxyMb content on the surface of the meat before and after blooming. The exposure to air allowed the oxygenation of the meat as evidenced from the content of OxyMb form that reached or exceeded (after longer storage inside the master bag) the value measured before the packaging. As it is possible to notice, the blooming time could progressively be decreased (down to 30 minutes) as storage of meat in the master bags was prolonged. This behaviour is in accordance with reports from Lindahl et al., 2006 who found that blooming was faster and more pronounced in meat aged for several weeks in vacuum packaging prior to exposure to air, owing to some loss of activity of the oxygen-consuming enzymes. The same Authors also reported formation of a deeper OxyMb layer. The depth to which the oxygen penetrates depends on a balance between oxygen concentration at the surface and tissue respiration, which consumes oxygen as it becomes available (Beggan et al., 2006). If the respiratory activity of meat is lowered during storage and oxygen is not consumed rapidly, blooming not only occurs more quickly but also is more obvious and the OxyMb layer is therefore thicker, typical of chill-stored meat on display under an oxygen-permeable wrap (Young et al., 1999). This interpretation is supported by the colorimetric data in Table 5.5 that lists values before and after blooming upon storage for 7 and 21 days in a master bag, and provides evidence that the colour indexes gained values similar or higher than those measured at the beginning of the storage (t_0).

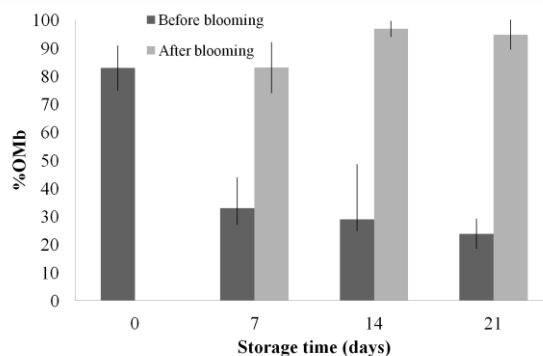


Figure 5.5. OxyMb content of beef meat, before and after blooming. The result at time 0 corresponds to meat before the packaging inside the master bag.

A crucial point in these anoxic packaging systems for the storage of meat cuts followed by blooming (Beggan et al., 2006) is the oxygen-transmitting ability of the overwrap film. Different Authors (Isdell et al., 1999; Beggan & Allen, 2004) suggested that a highly oxygen-permeable overwrap film (with OTR values around $20000 \text{ cm}^3 \text{ 24h}^{-1} \text{ m}^{-2} \text{ bar}^{-1}$ at 23°C , 0%RH) allows the achievement of blooming, even if commercially available lidding films lacked the right oxygen permeability especially at low display temperatures. In the experiment with steaks of *Longissimus lumoborum*, Beggan et al. (2006) estimated that a lidding film suitable for use in a ultralow-oxygen packaging system would require a permeability allowing 10-20% oxygen into the retail pack within 1 hours at approximately 4°C . In our work, the blooming was promoted by the presence of holes on the surface of the PVC stretch film. In this case, the perforation facilitated the gas exchange and the oxygenation of the product, even if a right modulation of perforations (in terms of dimensions and distribution) could be required to optimize the blooming and also for hygienic reasons. Also, perforation could be not required if the wrapping film ensures the full re-oxygenation of surface pigments.

Table 5.4 lists %OxyMb and colour indexes values before and after blooming upon storage for 7, 14 and 21 days in a master bag. The oxygenation of the meat surface was dependent on storage time of meat in the master bags ($p < 0.05$) as evidenced from the content of OxyMb form that exceeded (after longer storage inside the master bag) the value measured before the packaging (value at time 0). Lindahl et al. (2006) found that blooming was faster and more pronounced in meat aged for several weeks in vacuum packaging prior to exposure to air, owing to some loss of activity of the oxygen-consuming enzymes with time post mortem. If the respiratory activity of meat is lowered during storage and oxygen is not consumed rapidly, blooming not only occurs more quickly but also the OxyMb layer is therefore thicker, typical of chill-stored meat on display under an oxygen-permeable wrap (Young et al., 1999).

This interpretation is also supported by the colorimetric indexes in Table 5.4 that provides evidence that the Chroma and Hue angle gained values significantly different ($p < 0.05$) than those measured at the beginning of the storage (t_0).

Storage in master bag (days)	OxyMb (%)	Chroma	Hue angle ($^\circ$)
0	89.3 (8.0) ^a	36.2 (2.3) ^a	18.7 (1.1) ^a
7 BB	33.0 (6.0) ^b	27.2 (1.6) ^b	17.2 (1.7) ^b
7 AB	83.0 (9.1) ^a	34.5 (1.5) ^a	22.5 (2.0) ^c
14 BB	29.0 (4.2) ^b	25.8 (1.9) ^b	15.6 (2.5) ^b
14 AB	96.8 (5.4) ^c	37.9 (0.6) ^c	19.1 (1.0) ^{ac}
21 BB	23.8 (5.4) ^b	25.8 (1.6) ^b	16.2 (1.1) ^b
21 AB	96.8 (2.5) ^{ac}	38.6 (1.2) ^c	19.4 (2.7) ^{ac}

Table 5.4. OxyMb level, Chroma and Hue angle of steaks stored in master bag with scavengers, before and after blooming (BB and AB, respectively). ^{a-c}Means of the same column with different letters differ significantly ($p < 0.05$). Standard deviations into brackets.

A crucial point in these anoxic packaging systems for the storage of meat cuts followed by blooming (Beggan et al., 2006) is the oxygen-transmitting ability of the overwrap film. Different

authors (Isdell et al., 1999; Beggan & Allen, 2004) suggested that a highly oxygen-permeable overwrap film (with OTR values around 20000 cm³ 24h⁻¹ m⁻² bar⁻¹ at 23°C, 0%RH) allows the achievement of blooming, even if commercially available lidding films lacked the right oxygen permeability especially at low display temperatures. In the experiment with steaks of *Longissimus lumoborum*, Beggan et al. (2006) estimated that a lidding film suitable for use in an oxygen-depleted packaging system would require a permeability allowing 10-20% oxygen into the retail pack within 1 hours at approximately 4°C. In our work, the blooming was promoted by the presence of holes on the surface of the PVC stretch film. In this case, the perforation facilitated the gas exchange and the oxygenation of the product, even if a right modulation of perforations (in terms of dimensions and distribution) could be required to optimize the blooming and also for hygienic reasons.

5.3.3 Display life

After blooming, the trays were stored in a display cabinet at 4±2°C for 48 hours. Meat never stored in master bags and packed in PVC wrapped trays in air was taken as reference (R) and its evolution during the display life was compared with the quality of meat after the storage for 7, 14 and 21 days in master bags and respective blooming.

Packaging system ¹	MB ₇						MB ₂₁				T			
	T	MB ₇	MB ₂₁	T	MB ₇	MB ₂₁	T	MB ₇	MB ₂₁	T	MB ₇	MB ₂₁	T	MB ₇
<i>Display time</i> ²	t ₀	t _{BB}	t _{AB-0} [*]	t _{BB}	t _{AB-0} [*]	S	t ₂₄	t ₂₄	t ₂₄	S	t ₄₈	t ₄₈	t ₄₈	S
Chroma	36.2 ^b	27.2	34.5 ^{ab}	25.8	38.6 ^b	p<0.001	34.1 ^a	36.9 ^{ab}	37.9 ^b	p<0.001	28.8 ^a	37.0 ^b	36.4 ^b	p<0.001
Hue angle (°)	18.7 ^a	17.2	22.5 ^b	16.2 ^a	19.4 ^a	p<0.001	21.5	21.3	18.4	NS	20.1	20.7	18.0	NS
OxyMb (%)	89.3 ^b	33.0	83.0 ^a	29.0	96.8 ^c	p<0.001	90.7 ^a	96.0 ^b	94.6 ^b	p<0.001	69.4 ^a	81.3 ^b	96.8 ^c	p<0.001
MetMb (%)	3.04 ^a	7.40	7.8 ^b	7.90	4.10 ^a	p<0.001	17.1	16.4	12.2	NS	14.4	15.1	13.2	NS
mgMDA kg ⁻¹	0.03	0.05	0.05	0.13	0.13	NS	0.14	0.16	0.18	NS	0.21	0.22	0.21	NS
MBC (log ufc g ⁻¹)	3.7	4.4	4.4	5.1	5.1		4.3	4.0	5.0		4.9	4.6	6.2	
LAB (log ufc g ⁻¹)	1.9	5.5	5.5	5.7	5.7		2.1	4.5	5.3		2.2	4.8	6.3	
<i>Pseudomonas spp</i> (log ufc g ⁻¹)	3.2	3.3	3.3	3.2	3.2		3.7	3.4	3.0		4.2	3.6	3.2	

¹T: Meat in PVC trays never stored in master bag; MB₇: Meat in PVC trays stored 7 days in master bag with O₂ scavengers; MB₂₁: Meat in PVC trays stored 21 days in master bag with O₂ scavengers

²t_{BB}: Before Blooming; t_{AB-0}: After Blooming. The indexes after blooming are taken as the starting point of the display life for meat stored 7 and 21 days in master bag

^{a-c}The statistical comparison is made at each display time (t₀ or t_{AB}, t₂₄ and t₄₈). The effects of storage are different (p<0.05) if they have no common superscript letters

Table 5.5. Quality and microbiological indexes evolution during the display life, after blooming (AB), of steaks previously stored for 7 and 21 days into master bags with scavengers. ^{a-d} Means of the same column with different letters differ significantly (p < 0.05). ^{A-B} Means of the same group (R, M_{7AB}, M_{14AB}, M_{21AB}) with different letters differ significantly (p < 0.05).

Table 5.5 shows the evolution of some quality and microbiological indexes during the display life. From a statistical point of view, the quality indexes of meat never stored in master bag were compared, at each display time (t_0 , t_{24} and t_{48}), with those obtained after 7 and 21 days of storage in master bag with the oxygen scavenger. In this case, the values after blooming (t_{AB-0}) were taken as the starting point.

At the beginning of the display time (R_0 , M_{7AB-0} , M_{14AB-0} , M_{21AB-0}) differences were evident among the samples in terms of Chroma and OxyMb content ($p < 0.05$). As discussed previously, meat stored in master bags for longer times (21 days) achieved the best oxygenation after blooming and maintained a constant level of OxyMb and colour indexes during the display life, without suffering oxidation, as indicated also by the low values of the secondary lipid oxidation products that passed from 0.13 after blooming to 0.21 mg MDA kg^{-1} meat after 48h of exposure to air in retail display after removal from the master bag. In general, for meat stored longer in master bag (M_{14AB} , and M_{21AB}) the time of exposure to air in retail display after removal from the master bag on the display had no effect on OxyMb and Chroma evolution ($p > 0.05$), showing the colour stability of the *Semitendinosus* muscle after the anoxic storage.

On the contrary, meat never stored in master bag highlighted the highest loss in OxyMb and changes in Chroma index and both OxyMb and Chroma values at longer display times (t_{48}) were significantly different from those measured at time 0 ($p < 0.05$). In this case, the oxygen consumption rate by muscle could not be decreased, due to the short time post mortem (9 days).

Microbiological analyses showed that the level of *Pseudomonas* spp. was maintained constant over the exposure to air in retail display after removal from the master bag on the display on meat stored up to 21 days in a master bag with scavengers. This is probably due to the residual effect of CO_2 present in the modified atmosphere during anoxic storage. During the display life of meat also the evolution of proteolytic events by SDS-PAGE technique was followed. Also in this case no differences were evident among the samples (data not shown), hence it is possible to hypothesize that no strong proteolytic events during both the storage and the display life were evident.

5.4 Conclusions

This study was able to demonstrate the potential advantages of an anoxic master bag packaging system for *Semitendinosus* meat beef. The presence of O_2 scavengers was crucial for the resolution of transient discoloration, maintaining oxygen at levels able to influence positively the MRA of the muscle. On the contrary, meat storage failed within 7 days without scavengers and a permanent discoloration was observed. Oxygen scavengers play an important role in keeping the oxygen partial pressure within critical limits, ensuring proper conditions for preservation and for complete blooming of the meat inside its primary package for storage time as long as 21 days. The scavenger performance in terms of absorption kinetics and absorption capacity at low temperatures and at low oxygen partial pressures is the key factor in optimizing the whole master bag system. Also during the retail display, the quality attributes of steaks from *Semitendinosus* muscle were maintained for 48h of exposure to air in retail display after removal from the master bag in wrapped trays.

The application of the electronic paramagnetic resonance resulted in the successful determination of the oxidative state of myoglobin heme iron during storage in low oxygen environment. Thus, the interconversion of the three main forms of myoglobin was monitored until the obtainment of the deoxygenated pigment.

Another important point is the oxygen permeability of the primary packaging (tray plus wrapping film or tray plus lidding films). In this perspective, the gas permeability of the materials becomes strategic when, at the beginning of storage, it interplays with the ability of the scavengers in

removing oxygen from the meat surroundings, and when rapid blooming of the meat is desired after the removal from the master bag. These packaging variables should be evaluated in depth in further studies, taking also into account the different sensitivity of muscles towards low oxygen environment.

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6. High VS low oxygen partial pressures: Sliced beef meat chemical and biochemical quality evolution.

6.1 Introduction

The role of food packaging is to protect foods from contamination and damage, to contain the food, and to provide information to the consumers (Coles, 2003; Marsh & Bugusu, 2007). Food packaging plays a crucial role especially in the preservation of perishable foods, such as fresh vegetables, fruits and meats. Many solutions has been developed in order to increase packaging effectiveness in maintaining the quality and safety of the food, but also to improve its sustainability and messaging role.

As regarding fresh meat products, the focus is still very high on the primary function of packaging, pursuing the prolongation of meat commercial life through different approaches and especially modified atmosphere packaging (MAP) (McMillin, 2008). It consist in the removal of the air surrounding the product, followed in some cases by its replacement thanks to the flushing of a specific gas mixture, composed by oxygen, nitrogen, carbon dioxide or their mixtures (Directive No 95/2/EC).

The use of high oxygen concentrations (>70%) in meat MAP solutions aims to maintain a bright red colour of the product to attract consumers (Jeremiah et al., 1972; Faustman et al., 1989), exploiting the saturation of meat pigments with this gas, and in particular the formation of OxyMyoglobin. In fact, myoglobin is the main pigment present in meat, and its oxidative state is responsible of its colour (Mancini & Hunt, 2005). Also, high oxygen concentration helps in inhibiting the formation of MetMyoglobin, which is the oxidised myoglobin form and gives a brown undesirable colour to the meat. However, the great amount of oxygen made available to the product, may improve lipid oxidation and protein aggregation, and reduce juiciness and sensory quality of meat (Faustman & Cassens, 1990; Jayasingh et al., 2002; Lund et al., 2007; Zakrys et al., 2008; Clausen et al., 2009; Singh et al., 2011; Zakrys-Waliwander et al., 2012).

Low oxygen packaging systems include vacuum and modified atmosphere solutions. The absence of oxygen helps in reducing all the oxidative phenomena and the increase of aerobic microorganisms. Moreover, anaerobic bacteria such as lactic acid bacteria grow and acidify the product, with a bacteriostatic effect. Between the two, MA solutions have the main advantage to comprehend the use of carbon dioxide as the active component of the gas mixture inserted in the package. In fact, the complimentary use of carbon dioxide provides the inhibition of microbial spoilage (Piergiovanni & Limbo, 2010), not only during the storage in the MA, but also after the opening of packages, thanks to its residual effect (Silliker et al., 1977). The deoxygenation of myoglobin is a drawback of the oxygen absence, since DeoxyMyoglobin gives to the product a purple-red colour that consumers don't associate with fresh meat: this will negative affect visual appreciation at purchase level (Taylor et al., 1990). This problem can be overcome using a master bag system: meat is stored in anaerobic atmosphere, thus reducing oxidative phenomena and aerobic microbial growth, and then it is sold in its oxygenated form to avoid consumer rejection.

In the present work, we compared the effects of the two MAP solutions on the quality of fresh beef meat: *i*) a previously optimized low oxygen master bag packaging system and *ii*) a case-ready high oxygen packaging solution.

6.2 Materials & Methods

6.2.1 Meat cuts

The following meat cuts were tested: sliced beef (Italian breed Piemontese) from *Semitendinosus muscle*, 1.2 cm thickness. The primal cut was stored under vacuum for 1 week before being cut and packaged.

6.2.2 Packaging systems

6.2.2.1 Master bag system

Piemontese beef slices were packed into non barrier self-absorbent EPS trays (220*130*20 mm) and wrapped with a high permeability stretch film PVC-based, microperforated. Each tray contained one slice, which weighted about 100-150 g. Two trays were inserted inside a nylon-based barrier master bag (58*43 cm, O₂TR <0.01 cm³m⁻² 24h⁻¹bar⁻¹ at 23°C) with two oxygen scavengers (FreshPax[®] CR 8, Multisorb Technologies). The master bag were sealed in modified atmosphere (30% CO₂ and 70% N₂) using a chamber machine (Multivac...), applying the following packaging conditions: automatic vacuum; 750 bar gas; 1.2 s sealing time. Meat was stored in the dark for 14 days at 0.5±0.5°C: master bag were opened after 7 and 14 days, then meat was allowed to reoxygenate (blooming) at 6±1°C and finally exposed onto a display case for 48 hours in order to simulate the display life at retail level. At each opening time, meat was analysed before (BB) and after (AB) blooming, and after 24 and 48 hours of display life.

Meat packed into permeable trays and never stored inside master bag was taken as a control and analysed at time 0 and after 24 and 48 hours of storage at 6±1°C.

6.2.2.2 Tray lid system with high oxygen MA

Piemontese beef slices were packed into barrier trays (180*250*30 mm) containing an absorbent pad. Each tray contained two slices for a total weight around 300-400g, and with a thickness around 1.2 cm. Trays were sealed with a barrier film (Cryovac Mirabella[®] DL150) after flushed with a modified atmosphere (80% O₂, balance CO₂) using a tray lidding machine (Multivac, DE). Trays were stored at 6±1°C for 8 days and analysed at time 0 and after 2, 6 and 8 days of storage.

6.2.3 Methods

6.2.3.1 Headspace analysis

Measurements of gas concentrations in the headspace of master bags and trays were carried out using a portable gas analyser (Pac Check[®] Model 333, Mocon, USA). The oxygen concentration was measured in the headspace of each master bag and each high oxygen tray before opening.

6.2.3.2 Myoglobin forms and colorimetric indexes determination

The estimation of myoglobin forms concentration and colorimetric indexes were performed using reflectance spectra of meat samples. Spectra were acquired as reported in paragraph 3.2.3.5, as well as the myoglobin form estimation. Spectra underwent further elaboration using dedicated software (Color...), to obtain L*, a* and b* values and calculate Chroma and Hue angle indexes. Parameters set for the calculation were Illuminant D65 and 2° observer.

6.2.3.3 Visual appearance

Refer to section 3.2.3.2.

6.3.2.4 Determination of reduced glutathione (GSH) through DTNB assay

Refer to section 5.2.3.9.

6.3.2.5 Two-dimensions Isoelectric Focusing (IEF)

IEF was carried out using a Ettan IPGphor II apparatus (GE Healthcare) using 7 cm IPG strips (pH range 3-10) and samples being applied overnight using the in-gel rehydration method. The reswelling solution contained 7 M urea, 2 M thiourea, 2% CHAPS (w/v), 65mM DTT, 2% IPG buffer pH 3-10, and a trace of bromophenol blue. After focusing, the IPG strips were maintained for 15 min in equilibration buffer (0.375 M Tris-HCl, pH 8.8, 6 M urea, 2% SDS, and 20% glycerol) containing 65 mM DTT, and were then run for further 15 min in the same solution except that DTT was replaced by 0.243 M iodoacetamide. The second dimensional separation was carried out with a Bio-Rad Protean II xi vertical electrophoresis system using 12% SDS-PAGE gels of 1.0 mm thickness. Strips placed on the vertical gels were overlaid with 1% (w/v) agarose in SDS running buffer (25 mM Tris-HCl, 192 mM glycine and 0.1% (w/v) SDS), and subjected to electrophoresis at 10 mA/gel for 30 min and 16 mA/gel until the tracking dye reached the anodic end of the gel. After separation in SDS-PAGE gels, the proteins were visualized by Blue Coomassie staining and scanned using a EPSON Perfection V500 Photo (EPSON).

6.3.2.6 Reverse phase liquid chromatography.

Homogenates of time 0 samples and samples stored for 8 days in HO packages and 14 days in low oxygen pp were deproteinated adding 1.2 mL of ethanol to 0.3 mL of extract, and kept 1 hour at -20°C. Then, samples were centrifuged for 50 min at 13000 rpm at 4°C (5415R Centrifuge, eppendorf, Germany). Supernatants were recovered, dried using a XXX and resuspended in 0.6mL milliQ water and 0.1% TFA. Reverse phase HPLC was carried out using a HPLC system (510 Pump and 717 Autosampler, Waters, USA) equipped with a C18 column (Symmetry 300Å, C18, 5µm Packing, 4.6x250mm, Waters, USA) and coupled with a UV detector (996 Photodiode Array Detector, Waters, USA). For each samples, 100 and 200 µl were injected. The elution was performed at room T, using milliQ water with 0.1% TFA as eluent A, and acetonitrile with 0.1% TFA as eluent B. A linear gradient (0.8 ml min⁻¹) was applied until reaching 60% B. After each analysis the column was cleaned in 100% eluent B, then equilibrated with eluent A. Elution chromatograms were acquired and processed at 280 nm using Empower (Waters, USA).

6.2.3.7 In vitro meat digestibility

In vitro pepsin digestion. Meat samples were manually minced, then 0.5g inserted into polypropylene test tubes and added of 5 ml of HCl 0.05N. Proteins were hydrolyzed by gastric pepsin (porcine stomach mucosa, EC 232-629-3, ref P7012, Sigma) at a 1:2000 pepsin:protein ratio for 60 min at 37 °C under mechanical stirring. In vitro pepsin digestion was terminated by the addition of 10% (final concentration) trichloroacetic acid after 30 and 60 min. Samples were then centrifuged at 13000g for 10 min, and the hydrolyzed peptide content in the supernatant was measured at 280 nm (Lambda 2, Perkin Elmer, Italy).

In vitro pancreatin digestion. Protein hydrolysis from pancreatin was preceded by pepsin digestion for 60 min at 37°C as described previously. Samples pH was adjusted about 8 adding TRIS 1M. Proteins were hydrolyzed by pancreatic enzymes (pancreatin from porcine pancreas, EC 232-468-9, ref P1625, Sigma) at a 1:20000 pancreatin:protein ratio for 180 min at 37 °C under mechanical stirring. Pancreatin digestion was terminated by the addition of 10% (final concentration) trichloroacetic acid after 60, 120 and 180 min. Samples were then centrifuged at 13000g for 10 min, and the hydrolyzed peptide content in the supernatant was measured at 280 nm.

In vitro evaluation of meat digestibility was carried out before and after a thermal treatment (cooking). The cooking was performed by putting the meat in a pan for the time necessary to reach a core temperature around 60±2°C.

6.3.2.8 Statistical analysis

The influence of different packaging system on meat quality indexes was compared statistically for significant differences ($p < 0.05$) using one way analysis of variance (Software Statgraphics Plus for Windows, v.4). Tukey's Honest Significant Difference (HSD) test was used to determine significant differences between individual means when the treatment effect was significant ($p < 0.05$).

6.3 Results & Discussion

The headspace composition of master bags and high oxygen (HO) trays was monitored at each time of analysis. Master bags flushed with anoxic gas mixture registered a residual oxygen content around 0.5% right after sealing, while after 7 and 14 days of storage the oxygen concentration was <0.05 %, thanks to the activity of the oxygen scavengers and the barrier properties of the master bag material against oxygen permeation. As regarding the carbon dioxide, a slight decrease from 30% to 25% was recorded during the storage, partially due to the permeation of CO₂ inside the trays' headspace, the gas dissolution into the meat (Gill, 1988) and a minimum loss through the master bag.

HO trays were received to the laboratory 12 hours after the packaging operations: the headspace composition resulted 73.6% O₂ and 22.5% CO₂. During the storage, oxygen decreased reaching around 70.7% after 8 days, while carbon dioxide concentration slightly increased to 23.7 %. Oxygen reduction in the first hours of storage is mainly due to the saturation of meat pigments, which is the objective of this packaging system, to the loss through the package and potentially to the microbial metabolism (O'Grady et al., 2000). Other authors obtained similar results from studies carried out on beef and lamb meats (Kennedy et al, 2004;Murphy et al., 2013): oxygen content decreased with increasing storage time, and carbon dioxide inversely increased. This phenomenon is also more evident when varying the gas headspace to meat ratio, from 2:1 to 1:1 or 0.5:1, probably thanks to the higher influence of the microbial contribution.

The effect of the two preservation solutions on meat colour was evaluated through the calculation of colorimetric indexes, Hue angle and Chroma. They respectively describe the overall hue of the product and the colour saturation (i.e. intensity of the colour). As reported in Table 6.1, Chroma remained constant at all storage time of samples stored in master bag, excepting at time 14+2, which corresponds to samples stored for 14 days in low oxygen partial pressure (pp) and subsequent 2 days of aerobic display life. Differently, samples stored in high oxygen partial pressure showed a decrease in colour saturation already after 2.5 days and showed further reduction at the end of the storage, due to pigment oxidation.

MB			HO		
days after packaging	Chroma	Hue angle (°)	days after packaging	Chroma	Hue angle (°)
0	25.3 (0.1) ^a	37.5 (0.1) ^a	0.5	27.5 (0.8) ^a	37.6 (0.8) ^a
7 (AB)	21.2 (1.4) ^a	39.7 (0.9) ^{ab}	2.5	23.9 (0.6) ^b	36.8 (0.2) ^a
8 (7+1)	20.6 (1.6) ^a	40.7 (1.5) ^{ab}	6.5	24.3 (1.9) ^{bc}	38.1 (3.9) ^a
9 (7+2)	23.2 (2.6) ^a	41.6 (1.8) ^b	8.5	22.9 (2.5) ^c	38.4 (0.2) ^a
14 (AB)	24.7 (1.2) ^a	40.0 (0.5) ^{ab}			
15 (14+1)	24.0 (0.5) ^a	40.7 (1.5) ^b			
16 (14+2)	19.6 (1.9) ^b	41.7 (0.9) ^b			

Table 6.1. Chroma and Hue angle measurements of meat stored in master bag (MB) for 7 and 14 days after blooming (AB) and during 2 days of display life (+1 and +2), and meat stored in high oxygen packages (HO) for 8 days. Within each column, means with different letters significantly differ ($p < 0.05$).

Chroma decrease during oxygen exposure has been reported by Vitale and coauthors (2014), who investigated the effect of vacuum storage (very low oxygen pp) on beef meat quality during display in high oxygen pp. They also reported a faster decrease of Chroma during display of beef meat in high oxygen atmosphere, after longer storage in an oxygen-free environment.

Hue angle (H°) showed slight changes in samples stored in low oxygen pp (MB) and almost no variation in meat packaged in high oxygen (HO) atmosphere (Table 6.1). The increase of H° is correlated to a loss of redness (i.e. lower a^* value), which is mainly due to the loss of oxygenated pigments. A recent study interestingly highlighted that the fluctuations in the relative amount of myoglobin forms in the surface layer of the meat have a significant impact on all colorimetric parameter, except on Hue angle (Karamucki et al., 2013). In fact, they concluded that H° depends on the variation of the amount of pigments penetrated by light in the surface layer of the meat, expressed as the chromatic component of total absorption of light at 525nm (Krzywichi, 1979; Lindhal et al., 2001).

Colorimetric evaluations stated in this paragraph are confirmed by scanning images reported here below, in Figure 6.1.

A primary goal of modified atmosphere packaging systems is to favour the colour stability of meat products, inducing a predominant specific myoglobin state. High oxygen MA solutions specifically want OxyMyoglobin to prevail, because of the fascinating colour it gives to the meat (Troy & Kerry, 2010).

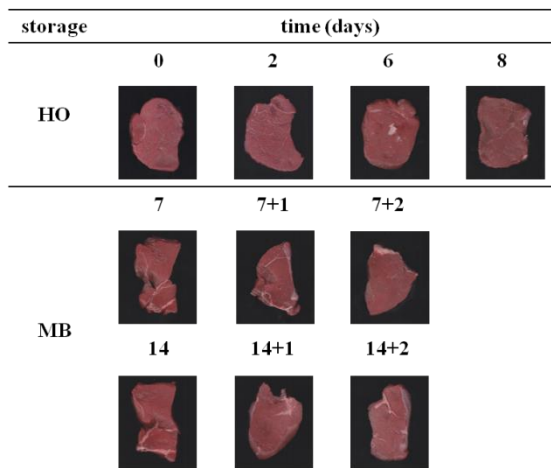


Figure 6.1. Digitalized images of samples stored in high oxygen (HO) for 8 days and samples stored for 7 and 14 days in master bag (MB). For the latter, images refer to different times of storage: time 7 or 14 days after blooming, and 24 (+1) and 48 (+2) h of display life.

At the beginning of HO storage (i.e. after 12 hours from packaging), meat presented a very high OMb content, around 93%, that decreased during the storage because of pigment oxidation (Table 6.2). In fact, MMb formation in sample stored in HO significantly increased already after only 2.5 days, and finally reached values around 19% after 8.5 days, reaching a border line situation for consumer acceptance and purchase decision (MacDougall, 1982).

MB			HO		
days after packaging	OMb	MMb	days after packaging	OMb	MMb
0	83.7 (2.9) ^a	3.9 (0.4) ^a	0.5	92.7 (3.5) ^a	1.2 (0.1) ^a
7 (BB)	33.7 (9.7) ^b	2.6 (1.5) ^a	2.5	86.7 (1.5) ^b	9.0 (2.2) ^b
7 (AB)	76.3 (4.4) ^a	6.5 (2.2) ^b	6.5	79.8 (0.7) ^b	11.1 (2.5) ^{bc}
8 (7+1)	79.7 (6.3) ^a	7.7 (4.1) ^b	8.5	77.8 (6.9) ^b	19.1 (7.7) ^{bc}
9 (7+2)	83.9 (2.8) ^a	5.3 (4.4) ^b			
14 (BB)	32.9 (11.2) ^b	2.5 (2.8) ^a			
14 (AB)	82.0 (3.8) ^a	2.1 (3.8) ^b			
15 (14+1)	86.1 (1.7) ^a	3.4 (3.2) ^b			
16 (14+2)	76.3 (3.9) ^a	16.6 (5.9) ^c			

Table 6.2. OMb and MMb concentration on meat stored in master bag (MB) for 7 and 14 days, before (BB) and after (AB) blooming and during 2 days of display life (+1 and +2), and meat stored in high oxygen packages (HO) for 8 days. Within each column, means with different letters significantly differ (p<0.05).

The pigment oxidation has been previously reported (O’Grady et al., 2000; Venturini et al., 2010) in samples stored in high oxygen MA for few days, but the real size of this phenomenon is difficult to monitor, since the thicker layer of OMb formed on the surface masks the development of an underlying MMb layer (Hood, 1984). In fact, because the higher sensitivity of DMb to oxidation than OMb, first MMb forms at the interface between the surface layer of oxygenated pigments and the central layer of deoxygenated pigments. Moreover, despite oxygen penetration inevitably causes myoglobin oxidation, the presence of reducing systems (i.e. NADH-dependent MMb reducing enzymes) results in its conversion into OMb, until the rate of MetMyoglobin formation overtakes the rate of its reduction. At this point, the MMb layer thickness increases with display life (Young & West, 2005).

The oxygen absence causes pigments deoxygenation (i.e. formation of DMb), as evident in Table 6.2: after 7 days in master bag and before the blooming (BB), OMb almost halved and MMb didn’t significantly change, hence DMb was the predominant form. This datum expresses the efficacy and effectiveness of the packaging system in ensuring an oxygen-depleted environment, thanks to the combination of proper packaging elements, such as MA composition and volume, gas barrier properties of packaging materials, and use of active devices (i.e. oxygen scavengers) (Stahl, 2007). In fact, since myoglobin deoxygenation occurs only through the transient formation of MetMyoglobin (Mancini & Hunt, 2005), and only at oxygen levels <0.05%, the removal of oxygen residual at time of packaging and the ability to maintain an ultra-low oxygen partial pressure are essential to ensure the preservation of meat quality.

Besides oxygen removal, the oxidative stability of meat results from the balance of its endogenous prooxidants (e.g. oxidized polyunsaturated fatty acids) and antioxidants (e.g. catalase, glutathione peroxidase, carotenoids), and the presence of components sensitive to oxidation (e.g. cholesterol, proteins, unsaturated fatty acids, pigments) (Decker & Xu, 1998; Surai et al., 2004a e b; Serpen et al., 2012; Jin et al., 2013).

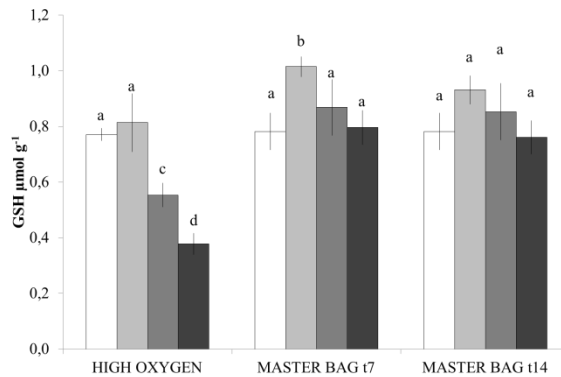


Figure 6.2. GSH concentration ($\mu\text{mol g}^{-1}$ meat) on meat stored in high oxygen packages (HO) for 8 days (0; 2; 6; 8) and meat stored in master bag for 7 and 14 days (0; 7; 7+1; 7+2 and 0;14;14+1;14+2). ^{a-b}Different letters indicate a significant difference ($p < 0.05$).

Glutathione (GSH) is the most abundant non-protein thiol present in animal cells, especially mammalian, and it is considered the main antioxidant able to provide reducing equivalents to the system (Sies, 1999; Surai et al, 2004b). In particular, its ability in scavenging superoxide anion, hydroxyl radical and hydroperoxides, makes it essential for the protection of cells against oxidative

attacks (Andersen, 1998; Lenzi et al., 2000; Arthur, 2000). GSH is also able to prevent the loss of protein thiols and vitamin E (Palamanda & Kehrer, 1993); in fact, a protective mechanism against protein thiols oxidation involving glutathione has been proposed by Lii and co-authors (1994), and it consist in the formation of reversible mixed-disulfide between thiols and GSH.

GSH analyses resulted in the evidence of a different behaviour of samples stored in HO packages in respect to samples stored in master bags with anoxic MA. The latter were able to maintain the same GSH content during the storage and also the following display life in air, while HO samples registered a significant decrease in GSH content after 6 and 8 days of storage (Figure 6.2). The evident decrease of GSH can be attributed to the high oxygen concentration in packages headspace; in fact, oxygen increases the oxidative stress, with protein thiols and lipids as the main targets. GSH acts as the first barrier to oxidative reactions, being oxidized instead of oxidation sensitive molecules, to form oxidized glutathione (GSSG). Oxidized glutathione can be further reduced back to GSH by glutathione reductase (GR) and NADPH (Andersen, 1998), to restore the optimal GSH/GSSG ratio, which ensure the cell reducing state (Schafer & Buettner, 2001). The greater formation of GSSG due to prooxidants causes greater activity of GR and oxidation of NADPH to obtain GSH. Thus, the higher oxidative stress leads to the consumption of the NADPH pool resulting from the pentose cycle, and, in a long term perspective, consequently limits the conversion of GSSG into GSH (Lii et al, 1996).

Oxidative reactions potentially occurring in meat products may target proteins, hence modifying their structure (Davies et al., 1987; Davies & Delsignore, 1987). Specifically, thiols and tyrosine are the most sensitive residues, being oxidized to form disulphide and dityrosine bonds (Gatellier & Santé-Lhoutellier, 2009), while N-terminal aminoacids undergo carbonyl addition (Berlett & Stadtman, 1997).

Two-dimensions electrophoresis (2DE) was performed in order to evaluate possible protein pattern changes during storage and eventual different behaviour in samples stored at different oxygen partial pressures. Homogenates from time zero samples and meat stored 8 days in HO packages and 14 days in low oxygen conditions (MB) were selected to explore the feasibility of this method. Whole homogenates were analysed, containing soluble proteins both from the myofibrillar and from sarcoplasmic protein fractions: the relative 2DE gels are presented in Figure 6.3.

2DE gels Blue Coomassie staining obtained from various samples were compared in order to identify possible macroscopic changes in the distribution of protein molecular weight and isoelectric point. In this frame, differences were observed in three zones, all at acidic pH, comparing samples stored in high or low oxygen partial pressure with time zero. As regarding HO packages (Figure 6.3 B), zones α and β differ from time zero samples and are characterised by a higher number of spots, thus suggesting an increase in high molecular weight (MW) proteins extracted from whole samples. According to reports by other authors, these spots were identified as tropomyosin, antioxidant enzymes and chaperone proteins and represent the main molecules present in this area (Hwang et al., 2005; Promeyrat et al., 2011; Joseph et al., 2012). Tropomyosin increase may be correlated to proteolytic processes that cause protein release from myofibrils, while an increase in antioxidant enzymes (e.g. selenium binding protein and mitochondrial/cytosolic superoxide dismutase) may be related to a response to oxidative stress, in this case due to the presence of very high oxygen pp (Renner et al., 1996). Chaperone proteins, as heat shock proteins, protect against protein denaturation, aggregation and fragmentation, and have been related to meat colour, due to their contribution in maintain pigments structure during meat aging (Sayd et al., 2006; Joseph et al., 2012).

Samples stored for longer time in low oxygen concentration (Figure 6.3 C) showed modest differences in the acidic zone of the gel, with a decrease of high MW spots (around 70-50 kDa)

and an increase in medium MW spots (around 45-30 kDa), with respect to the sample at time zero. The relative distribution of the proteins in zone γ in samples at low oxygen partial pressure was different from the proteins observed in control samples. The more intense spot present in each gel in this zone may be identified as actin, and acidic proteins focused on the same lane might be its isoforms or fragments (Hwang et al., 2005). Thus, changes in the γ zone could be correlated to an increase in actin release from the myofibrillar structure. Another hypothesis is that this protein may be derived from the degradation of tropomyosin (zone α) as a consequence of storage. A mass spectrometry analysis will clarify the nature of this protein.

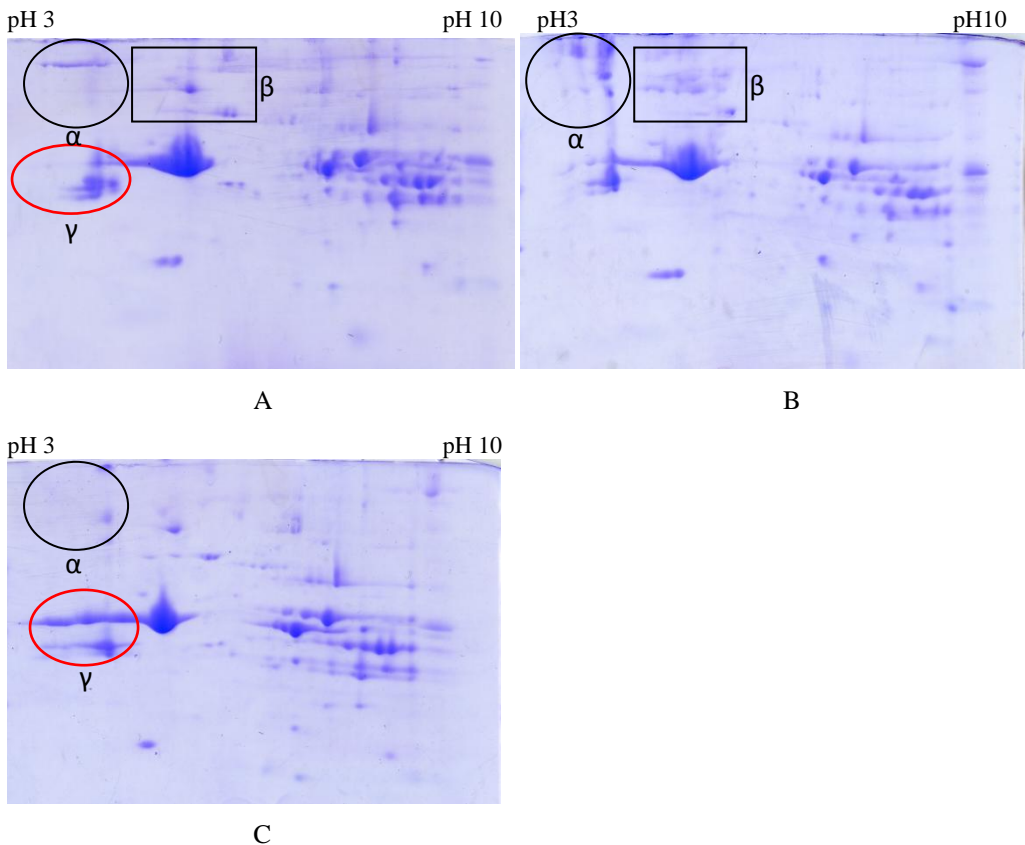


Figure 6.3. 2DE gels of samples at time zero (A), after 8 days of storage in HO (B) and 14 days in low oxygen pp (C).

The analysis of the peptidic fraction of meat homogenates through reverse phase chromatography did not show significant differences in various samples, suggesting that proteolytic event are similar in both high and low oxygen storage environments during storage. In fact, it was reported that the major protease systems do not degrade proteins to their constituent amino acids, but result in a modest myofibrillar degradation that facilitated dissociation into its main components (i.e. single proteins) (Huff Lonergan et al., 2010).

Protein modifications resulted from oxidative phenomena influence their functionality and the exposition of peptidic bonds to proteases, thus possibly affecting protein digestion, which is of great interest in the evaluation of the nutritional quality of meat products. *In vitro* digestion was performed on all samples, before and after cooking treatment, to assess the accessibility of peptidic bonds to proteases after meat storage with different modified atmospheres and to evaluate the influence of the cooking treatment on meat digestion.

Raw meat *in vitro* digestion (Figure 6.4, left panel) presented an increase of the released peptide content with protease action time. The first hour of treatment only pepsin action was considered, then pancreatic enzymes completed the digestive process. The data presented in Figure 4.6 suggest that meat digestibility was higher at time 0. The lowest formation of hydrolysed peptides was detected in samples stored 6 days in HO packages and the value was almost half of the amount of time zero samples. As reported by other authors (Seyfert et al., 2005; Lund et al., 2007; Bax et al., 2012), the storage in aerobic environment and even more in high oxygen atmosphere promotes protein oxidation and aggregation, thus reducing the accessibility of specific AA residues to proteases. Protein aggregation can also affect the sensory quality of beef, especially in terms of tenderness and texture (Rowe et al., 2004). Differently, meat stored in master bag in anaerobic conditions presented a lower decrease of meat digestibility after 7 and 14 days, compared to high oxygen packages, even if storage time was longer. A possible explanation has been proposed by Santé-Lhoutellier and coauthors (2008), when studying the effect of diet and meat storage on lamb meat protein oxidation and digestibility. The protein susceptibility to protease attack seems to change differently depending on the levels of oxidation to which meat is exposed (Davies, 2001; Grune et al., 2004). In fact, low levels of oxidation likely results in modest modification in protein structure, leading to a greater recognition by proteases and to a consequent increasing digestibility. On the contrary, high level of oxidation are characterised by the formation of aggregates, which modify protein chemical and physical properties and a decrease in proteolytic susceptibility. This results in an accumulation of non-hydrolysed proteins in human goat, where the colonic flora is able to ferment it into mutagenic products, such as phenols and p-cresol, increasing the risk of colon cancer (Evenepoel et al., 1998). Another side effect of decrease in protein hydrolysis is the decrease of meat tenderness, as reported by Clausen et al. (2009) and Lund et al (2007). In their works, the loss of tenderness occurring after high oxygen MAP (70-80 % of oxygen) in beef meat was found to be associated with a decrease of the rate of the myofibrillar fragmentation - a useful indicator of proteolysis -, compared to samples stored in air (about 20% oxygen) or anaerobic conditions.

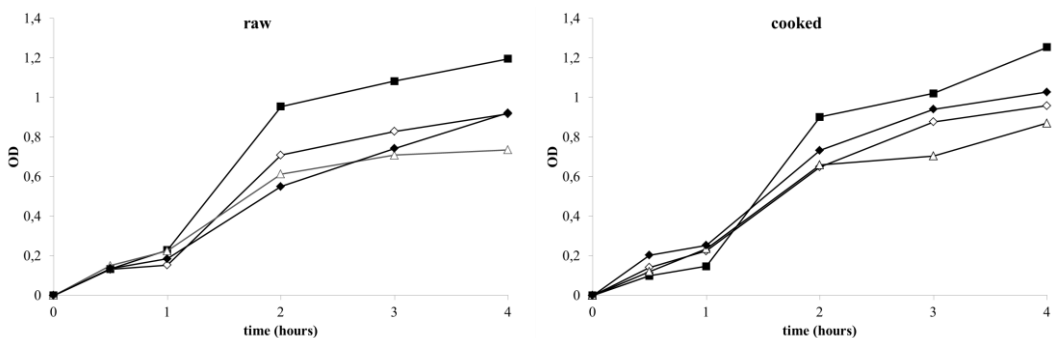


Figure 6.4. *In vitro* digestibility of raw (left) and cooked (right) meat stored in high oxygen packages (HO) for 6 days (△) and meat stored in master bag (MB) for 7 (◇) and 14 (◆) days during *in vitro* digestion, compared with control samples at time 0 (■).

Meat generally undergoes a thermal treatment before being eaten: the cooking process accelerates oxidation because of the increase of free radical production and the simultaneous decrease of antioxidant protection (Gatellier et al., 2010). Meat stored both in high or low oxygen systems presented a slight increase in the absorbance at 280 nm (Figure 6.4, right panel), correlated to a larger amount of hydrolysed peptides, thus linking to a greater digestibility of the product. These results are in accordance with those obtained by Bax et al. (2012), who investigated the effect of different cooking temperatures on in vitro digestion of pork meat. In their study, they found that cooking temperatures below 100°C positively affect protease activity. In fact, after heat treatment of sample they observed an increase of exposed hydrophobic portions of proteins, that promotes the accessibility of proteases, and in particular pepsin, to cleavage sites. This phenomenon occurs only until a certain heating temperature, as at higher treatments the aggregation process appears to overtake the thermal denaturation process, thus reducing protease accessibility.

6.4 Conclusions

The comparison of two different oxygen partial pressures for the preservation of sliced beef meat was pursued in this study. A previously optimized master bag packaging system was selected as the low oxygen solution, and a try lid system containing 80% oxygen in its headspace as the high oxygen one. The former showed the higher colour stability for the longer storage time, as well as it registered better biochemical and nutritional properties, in terms of reduced glutathione content and protein digestibility (before and after cooking).

All together, this investigation suggest that in the two packing system (high and low oxygen pp) two different overall protein organization are present.

Further investigations are needed to determine which are the specific protein involved in these structural changes, as well as their relation with the oxidative reactions occurring to the lipid fraction.

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7. Preliminary study on low oxygen packaging systems for horse meat

7.1 Introduction

Meat is a relevant component in the human diet thanks overall to its protein content: in fact, meat is considered the first source of protein, followed by fish, eggs and legumes (Weder & Belitz, 2003; Whitney & Rolfes, 2008).

Most common animals bred for meat production are beef, pig, chicken and turkey. Meat consumption strongly depends on historical and cultural habits, not only regarding the amount of product consumed, but also the kind of meat. India and Brazil are the biggest producers of bovine livestock, while United States and Europe are the main consumers. On the contrary, China and Europe are the leaders both in the production and the consumption of pork meat (USDA, 2012). Apart from those breedings, many other animal species provide valuable meats, but their market is generally confined by geographical or cultural borders. A good example for the latter situation is horse meat. In fact, although it is a high quality meat product, it's very hard to enlarge its market because many people still see horses as pets and many countries don't allow horse slaughter. In the centre Asia, horse meat is considered the most prestigious meat (Ferret, 2010), consequently, the production and consumption are very relevant phenomena. In Europe, the biggest horse meat producers are Italy, Poland, Spain and France, covering totally about 85% of European horse slaughtered (Eurostat, 2007). Almost all these states import most horses from other EU member states (Istat, 2010) or third countries, such as Canada, Argentina, Brazil, Mexico and Uruguay (HSI, 2012). On the other hand, horse meat is seen as a taboo in many countries (e.g. Russia, United Kingdom and Ireland) or it is not consumed for religious reasons (e.g. Jewish). A peculiar case is represented by the USA, which just abrogated the American Horse Slaughter Prevention Act (H.R. 503/S. 311) at the end of 2011, leading to the possibility to re-open for American slaughter houses. This decision has not been welcomed by many people, because of their sensitivity towards horses as pets, enabling them to reflect on horse meat as a high nutritive food. Because of this sensation is diffused in some areas all over the world, the research carried out on this topic is limited and fragmented.

In general, horse meat is characterised by a high content of water (74.51%), proteins (21.44%) and glycogen, in comparison with other fresh meats (Martin-Rosset, 2001; Znamirowska & Stanislawczyk, 2005). Moreover, horse meat is considered a high quality meat product thanks to its content of essential aminoacids, unsaturated fats, vitamins and minerals (Badiani et al. 1997; Lenis et al., 1999; Lombardi-Boccia et al., 2005; Lee et al., 2007; Lorenzo et al., 2010; Oshima, 2002; Boselli et al., 2012). In particular, Del Bo' and colleagues (2013) investigated the effect of moderate horse meat consumption on iron status, lipid profile and fatty acid composition of red blood cells in humans, concluding that the regular consumption of horse meat may contribute to the dietary intake of n - 3 polyunsaturated fatty acids and may improve lipid profile and iron status in healthy subjects.

The storage of horse meat requires a strictly anoxic environment because of its high oxygen sensitivity, which causes a fast and irreversible oxidation of the product surface. Hence, packaging solutions imply the use of barrier materials, in combination with vacuum or low oxygen MAP. The increasing interest of meat packers and retailers on the use of bio based or biodegradable materials, leads to the investigation of the efficacy of these materials when substituting barrier petrol-derived packages in MAP systems. In fact, although standard materials come from a non-renewable source (i.e. petrol), their performances in terms of barrier properties have been perfectly optimized using different material combinations and processes. On the contrary, bio based materials production is

based on renewable resources, but generally lacks some mechanical or physical properties, or need extended processing to achieve them. As an example, polylactic acid (PLA) is produced through the polymerization of lactic acid coming from fermentative processes using bacteria, which are evidently a renewable resource. However, its commercial formulations so far cannot ensure very high barrier properties against gas permeation, thus making difficult its implementation in the MAP application for foods.

The aim of this work was the evaluation of the effects of different packaging solutions on the quality of sliced horse meat. In particular, we investigated the possibility of substituting the barrier primary packages with non-barrier ones, thanks to the improvement of the gas barrier property by means of an active packaging solution. Non barrier primary materials selected were made by polylactic acid (PLA): foamed trays were obtained from a foil of expanded PLA laminated with a rigid PLA film, and lid films were made on PLA with a barrier coating.

The work has been divided into two parts: the first focused on the comparison of standard and non-barrier primary packages containing modified atmosphere, with and without an oxygen scavenger; the second part focused on the use of a master bag packaging system, inserting standard and non-barrier primary packages into barrier bags containing modified atmosphere and oxygen scavengers.

7.2 Materials & Methods

7.2.1 Meat cuts

Primal cuts of horse meat were obtained from Argentina, stored in vacuum pouches for about 4 weeks. Meat was sliced at an industrial packer, and packed within 20 minutes from primal cuts exposure to air.

7.2.2 Packaging systems

Horse meat slices were packed in barrier primary packages containing modified atmosphere (55% CO₂, 45% N₂). In particular, two trays were used, both in the B5 size (230*150*40 mm) and with an absorbent pad: i) yellow barrier EPS tray, with a O₂TR < 10 cm³ day⁻¹ pack⁻¹, 23°C e 0% RH; ii) white expanded PLA tray laminated with a rigid PLA film to enhance the barrier properties of the tray itself (named XPLA). Lid films used were: i) barrier laminated red film, with a O₂TR < 3 cm³ m⁻² day⁻¹ atm⁻¹ 23°C 0% RH; ii) non laminated coated PLA (PLA + oxygen barrier coating), O₂TR 2.26 cm³ m⁻² 24h⁻¹ atm⁻¹ at 23°C and 85% RH (Oxaqua, Metalvuoto); ii) non laminated coated PLA (PLA + silicon oxide coating), O₂TR 14.07 cm³ m⁻² 24h⁻¹ atm⁻¹ at 23°C and 85% RH (Ceramis® CPN 003, Amcor).

Active packaging solutions were tested, using different oxygen scavengers (Multisorb Technologies Inc.):

- OS A: FreshMax® 30. Adhesive label with a water proof and gas permeable top, and a nominal capacity higher than 20 cc O₂.
- OS 1: FreshPax® CR 4. Sachet made on a high permeable material and a nominal capacity higher than 200 cc O₂.
- OS 2: FreshPax® CR 4. Sachet made on a water proof and high permeable material, and a nominal capacity higher than 200 cc O₂.

- OS 3: FreshPax® CR 8. Sachet made on a high permeable material and a nominal capacity higher than 600 cc O₂.
- OS 4: FreshPax® CR 30. Sachet made on a high permeable material and a nominal capacity higher than 1000 cc O₂.

The testing was divided into two parts. Here below the table (Table 7.1) that summarizes the time of analysis of each packaging solution is reported.

Packaging solution	Time of analysis (days)
PART 1	
A	0 ^(*) , 5, 8, 12, 15, 19, 22, 26, 29
B	0 ^(*) , 5, 8, 12, 15, 19, 22, 26, 29
PART 2	
A (A1-A2-A3)	0,1, 5, 11
B (B3-B4-B5-B6)	0, 1, 4, 6, 11, 15
C	0, 6, 6+1, 6+2, 11, 11+1, 11+2, 18, 18+1
D	0, 11, 20, 29

Table 7.1. Time of analysis of all packaging system tested for sliced horse meat.
(*) time zero samples analyzed 4 hours after packaging.

First part:

- A. EPS tray + red barrier film, with or without one oxygen scavenger type OS A inside the tray.
- B. Expanded PLA tray + PLA laminated lid film, with or without one oxygen scavenger type OS A inside the tray.

Second part:

- A. Expanded PLA tray + PLA laminated lid film, with or without one oxygen scavenger inside the tray.
 - A1. With one oxygen scavenger, type OS 1;
 - A2. With one oxygen scavenger, type OS 2;
 - A3. Without any oxygen scavengers.
- B. Expanded PLA tray + PLA laminated lid film. Two trays were inserted inside a barrier master bag, flushed with the same gas mixture of the trays (55% CO₂, 45% N₂), with or without oxygen scavengers inside the tray and/or the master bag itself.
 - B3. One oxygen scavenger type OS 1 inside the tray and three oxygen scavengers type OS 3 inside the master bag.
 - B4. One oxygen scavenger type OS 1 inside the tray and no scavengers inside the master bag.

- B5. No oxygen scavengers inside the tray and three oxygen scavengers type OS 3 inside the master bag.
- B6. No oxygen scavengers inside the tray neither inside the master bag.
- C. Barrier EPS trays in which the barrier layer has been removed on the bottom and partially on the sides + barrier red lid. Two trays were inserted inside a barrier master bag with one oxygen scavenger OS 4. Both the trays and the master bag were flashed using the same MA (55% CO₂, 45% N₂).
- D. Barrier EPS trays + barrier red lid film. Two trays inserted inside a barrier master bag without any oxygen scavenger and flushed with MA composed by 50% CO₂ and 45% N₂.

Sample packed using the solution A of the first part of the test were considered the control samples for the second part of the test.

7.2.3 Methods

7.2.3.1 Headspace analysis

The evolution of the headspace gas composition was monitored using a portable gas analyser (Check Mate 2, PBI Dansensor), able to detect oxygen and carbon dioxide concentration.

7.2.3.2 Colour evaluation and visual appearance

For method description refer to section 3.2.3.2, except for the calculation of ΔE , which indicates the difference between the sample and a reference. This index was calculated using the following formula:

L^* and L^*_0 represent the Lightness of the sample and of the control respectively; the same format has been used for a^* (red-green) and b^* (yellow-blue).

7.2.3.3 pH

Refer to section 3.2.3.3.

7.2.3.4 Microbiological analysis

Aerobic mesophilic bacterial count and lactic acid bacteria were monitored as described in section 3.2.3.4.

7.2.3.5 Reflectance spectra

As regarding the monitoring of myoglobin oxidative states, a different approach was used in this test. Reflectance spectra were acquired as described in section 3.2.3.5, then a qualitative evaluation

was carried out, comparing the spectrum of each sample with the spectra of myoglobin pure forms. The evolution of myoglobin oxidation was also described through the calculation of the 630/580 nm ratio (AMSA, 1991).

7.2.3.6 Determination of reduced glutathione (GSH) through DTNB method

Refer to section 5.2.3.9.

7.2.3.7 Statistical analysis

Refer to section 5.2.3.8.

7.3 Results & discussion

7.3.1 Part 1

During the first part of the work, horse meat slices were packed into XPLA trays with a barrier PLA lid, while control samples were inserted into barrier EPS trays sealed with a barrier lid. Both solutions were combined with the insertion of the same low oxygen modified atmosphere, rich in CO₂, and oxygen scavengers.

During the storage at 0±0.5°C, headspace analyses showed that inside standard packages (Figure 7.1, left) the oxygen was always <0.002% independently on the presence of the oxygen scavengers. Hence the barrier material and the gas flushing allow the maintenance of the anoxic environment throughout the whole storage.

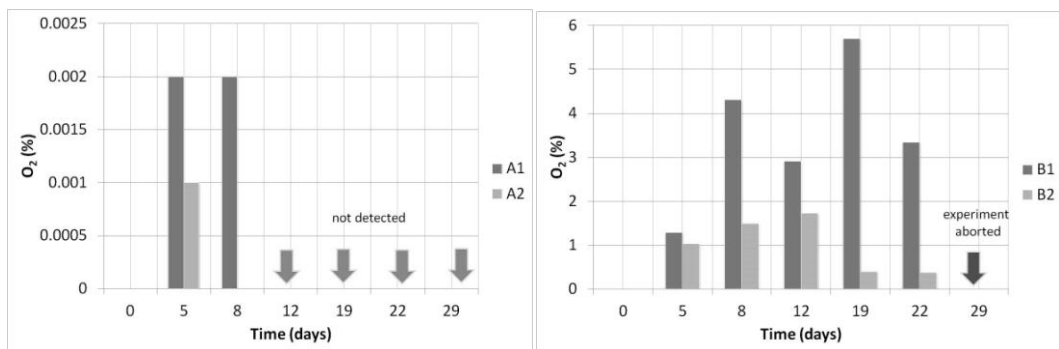


Figure 7.1. Evolution of the oxygen content in the headspace of standard trays, without and with oxygen scavengers (respectively A1 e A2; on the left), and PLA trays, without and with oxygen scavengers (respectively B1 e B2; on the right).

The presence of the scavengers helps in further reduction of the oxygen level to undetectable levels after 8 days of storage, but in the following days no differences were recorded between the two solutions. In PLA packages (Figure 7.1, right) the oxygen reached about 5% when the oxygen scavenger was absent, while it remains <1.8% in the presence of the active device. Hence, the PLA favours the ingress of oxygen, causing a significant change in the headspace composition and consequently the degradation of the product, which is very sensitive to the oxygen. In fact, it is well

known that low oxygen modified atmosphere packaging solutions preserve the quality and safety of case ready horse meat (Ribezzo et al., 2002).

As regarding the carbon dioxide evolution in standard packages (Figure 7.2 left), the initial content of around 60% slightly decrease at 50% after 5 days of storage, but it remains constant until the end of the test, with no evident differences between samples with and without the oxygen scavenger. Hence, the packaging materials are able to prevent not also the oxygen permeation inside the package, but also the CO₂ loss in the external environment. Packages made by PLA materials (Figure 7.2, right) are characterised by a gradual and strong reduction of the CO₂, that is almost halved after only 5 days of storage and reached values <15% in the last days of the test. This loss is caused by the high CO₂ permeability of the materials: the lid has a PCO₂ around 1000 ml m⁻²24h⁻¹ (1 atm and 85% UR).

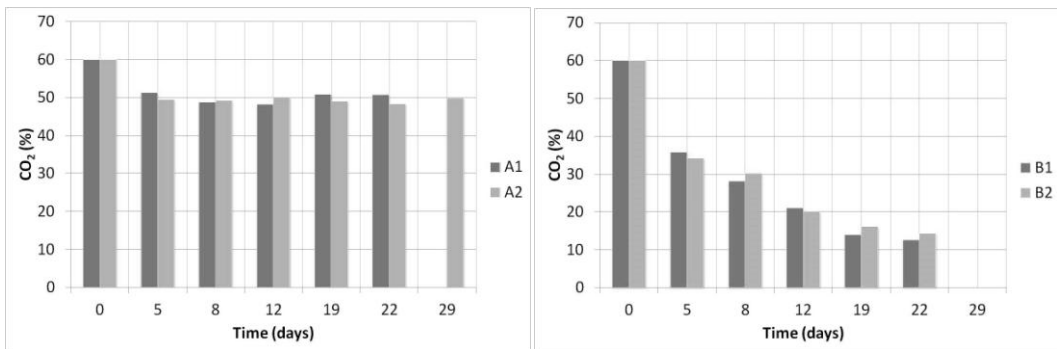


Figure 7.2. Evolution of the carbon dioxide content in the headspace of standard trays, without and with oxygen scavengers (respectively A1 e A2; on the left), and PLA trays, without and with oxygen scavengers (respectively B1 e B2; on the right).

CO₂ is included in modified atmosphere especially because of its efficacy in inhibiting microbial growth (Jay, 2000; Piergiovanni & Limbo, 2010), in fact CO₂ dissolves in the medium forming carbonic acid, that acidifies the food: this process is responsible for the inhibition of microbial growth and cellular respiration, thus slowing product degradation. The CO₂ concentration needed in the package depends mainly on the product itself and on the storage time. The excessive loss of the gas through the packaging materials may cause the obtainment of gas concentration too low to guarantee its efficacy; hence the selection of the material is strictly correlated with its barrier properties.

Microbiological analyses were carried out at specific times during the storage of samples, and in particular the total aerobic mesophilic count (TBC) and lactic acid bacteria (LAB) were evaluated, as long as pH measurements. Samples packed with standard trays and lids (data not shown), were characterised by an increase of TBC and LAB after 5 days of storage, then both stabilized around 10⁷ ufc g⁻¹ until the end of the test. Same values were reported by other authors (Brindani, 2003; Franzetti et al., 2003). The pH consequently decreased, due to the production of lactic acid by the microorganisms.

Colorimetric measurement carried out during the test allowed the calculation of Hue angle and Chroma that are indexes useful for the evaluation of fresh meat appearance. Hue angle (H°) describes the overall hue of the product, expressing the similarity or difference with one of the unique hues (yellow, red, blue and green) and assuming values between 0° and 360°. In the case of

horse meat, hues ranging from purple-red to brown are expected as long as the deterioration of the product occurs. As reported in Figure 7.3, samples packed with the standard materials maintain a stable H° value around 10° for 29 days of storage. The presence of the oxygen scavenger doesn't show any influence on the variation of the hue of the product for the whole storage.

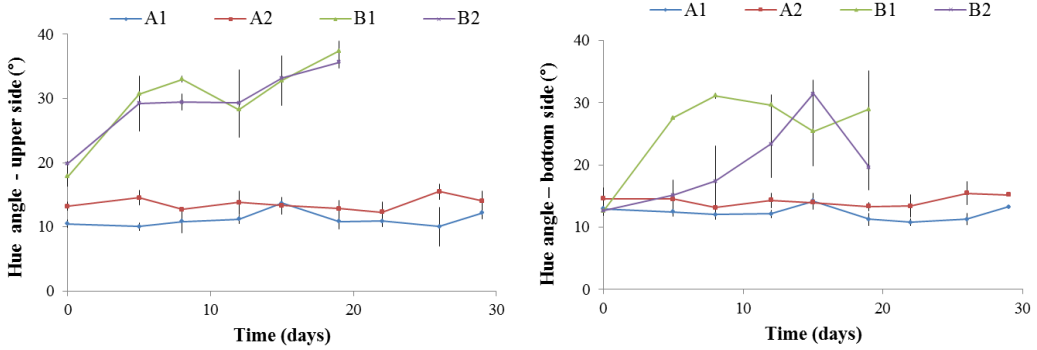


Figure 7.3. Evolution of Hue angle of the upper (left) and bottom (right) side of horse meat slices placed into standard (without and with oxygen scavengers, respectively A1 e A2) and PLA packages (without and with oxygen scavengers, respectively B1 e B2).

Samples stored in PLA packages showed a different H value already at the beginning of the storage, in fact, time zero analyses were carried out after 3 hours from the packing. The ingress of elevated quantities of oxygen in the headspace of the primary packages caused a rapid and evident discoloration of the meat, independently on the presence of the active device.

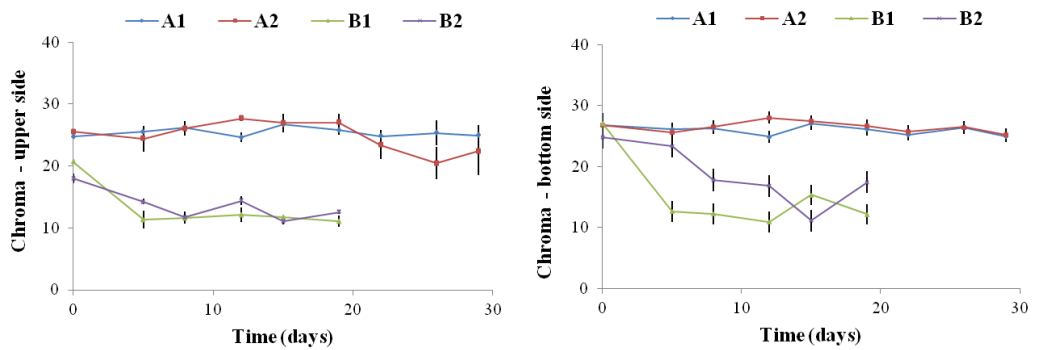


Figure 7.4 Evolution of Chroma of the upper (left) and bottom (right) side of horse meat slices placed into standard (without and with oxygen scavengers, respectively A1 e A2) and PLA packages (without and with oxygen scavengers, respectively B1 e B2).

Figure 7.4 reports the evolution of Chroma of the same samples during the refrigerated storage. Chroma describes the colour saturation and mainly depends on the myoglobin redox state. Samples

stored in the standard packages show a value around 26 that remains constant until the end of the test, while samples stored in PLA packages are characterised by a lower Chroma already at the beginning of the test and undergo a strong decrease of the index after only 5 days.

The increase of H° and the decrease of Chroma are mainly correlated to the reduction of a^* values (data not shown), that is the index of redness: during the storage it decreases because of the conversion of OxyMyoglobin into MetMyoglobin, due to the increase of oxygen in the headspace. Thus, PLA materials did not show the necessary barrier characteristics needed to guarantee the maintenance of an anoxic environment. Furthermore, the selected oxygen scavenger was not appropriate to reach the objective.

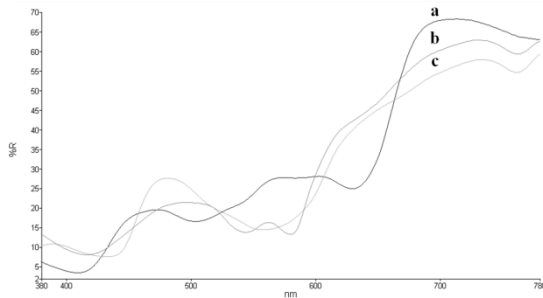


Figure 7.5. Reflectance spectra of pure myoglobin forms MMb (a), OMb (b) and DMb (c), obtained from fresh horse meat slices.

Fresh meat colour is given by the redox state and ligands of myoglobin, the predominant pigment. For the determination of these states, a spectrophotometric method is adopted (AMSA, 2012), that implies the preparation of pure forms for the three main forms of myoglobin detectable in fresh meat products: OxyMyoglobin, MetMyoglobin and DeoxyMyoglobin. The pure forms are obtained through specific treatments of fresh meat samples, and their reflectance spectra are reported in Figure 7.5.

OxyMyoglobin (OMb) is the predominant form in oxygenated fresh meat, in fact it is the reduced and oxygenated form that gives a bright red colour to the meat. DeoxyMyoglobin (DMb) is either a reduced form, but has no ligands and characterises deoxygenated meat (e.g. stored under vacuum or low oxygen MAP) by a purple red colour. Finally, MetMyoglobin (MMb) is the oxidised form, which concentration increase with meat ageing due to the degradation and oxidation of the product; it gives a brown red colour to the product.

Since samples are stored in low oxygen MAP, it is expected to record deoxygenated pigments during the storage and a gradual increase of MetMyoglobin close to the end of the presumptive shelf life, because of the product degradation.

In Figure 7.6 the spectra of all samples at t_0 and after 8 and 19 days of storage are reported. Samples stored in standard packages presented a spectrum similar to the DMb one, while samples stored in PLA packages show a great difference already at time 0. Main differences are evident around 540-580 nm, where samples B1 and B2 present higher reflectance values, and 610 nm where the same samples have a lower reflectance: these changes may be attributed to the formation of MMb on the product surface (AMSA, 2012; Mancini et al., 2005), leading to a brownish colour, in agreement with colorimetric data. Hence, the PLA solution here investigated is not able to preserve meat quality, and the preferable repackaging system remains the standard solution using barrier materials.

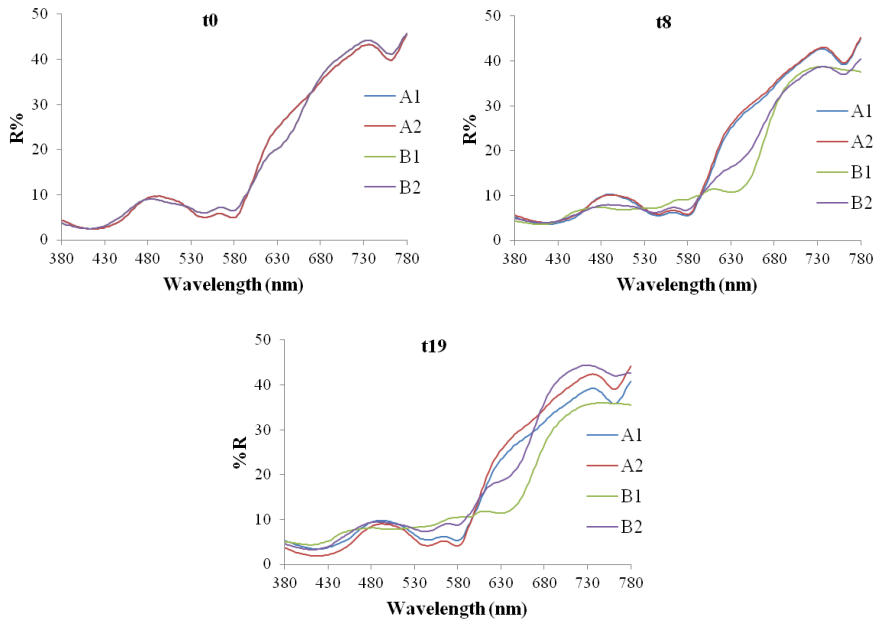


Figure 7.6. Reflectance spectra of horse meat slices placed into standard (without and with oxygen scavengers, respectively A1 e A2) and PLA packages (without and with oxygen scavengers, respectively B1 e B2), and stored for 0, 8 and 19 days.

Reflectance spectra were further elaborated, following the AMSA guidelines (AMSA, 1991; AMSA, 2012), that suggest to calculate the 630/580 nm ratio to evaluate the behaviour of myoglobin oxidation. In fact, this ratio is considered an indicator of the colour change of meat caused by MetMyoglobin formation.

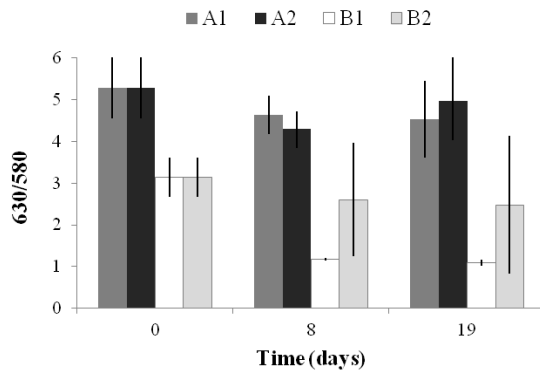


Figure 7.7. 630/580 ratio evolution of horse meat slices placed into standard (without and with oxygen scavengers, respectively A1 e A2) and PLA packages (without and with oxygen scavengers, respectively B1 e B2), and stored for 0, 8 and 19 days.

Values closer to 5 correspond to the presence of high percentages of reduced pigments (with slight differences between OMB and DMb) while the decrease to values close to 1 is correlated to the accumulation of MMb (Fernandez-Lopez et al., 2000). As evident in Figure 7.7, samples A1 and A2 maintain a ratio around 5 for the whole storage, hence their surface is rich on deoxygenated pigments, possibly DMb thanks to the anoxic environment inside the package. On the contrary, samples stored in PLA packages presented lower values from the beginning of the storage, until the end at day 19. This is due by the increase of oxidized pigments (MMb) that causes meat discoloration: the predominant colour of the sample is brown-red.

Scanning images of all samples are reported in Figure 7.8: as evident, samples B1 and B2 present a brownish appearance at time 0, which is only 3 hours after packaging operations, while samples A1 and A2 maintain a nice purple colour until day 29.

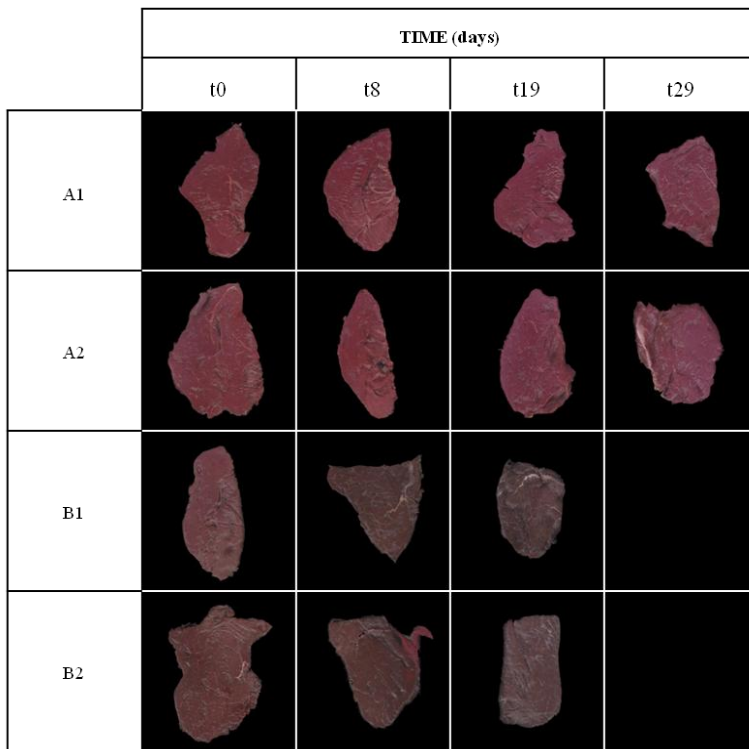


Figure 7.8. Scanning images of horse meat slices placed into standard (without and with oxygen scavengers, respectively A1 e A2) and PLA packages (without and with oxygen scavengers, respectively B1 e B2), and stored for 0, 8, 19 and 29 days.

The DTNB assay for the detection of reduced glutathione (GSH) was performed on meat extracts from samples stored in both standard and non-barrier packages. Since no differences were detected between samples stored using the same packaging materials, with or without oxygen scavengers, results are presented as the average of the two combinations. Samples stored in the standard package (A), showed a slight decrease of GSH while samples stored in the bio-based packages recorded a greater reduction after only 5 days (Figure 7.9). We can hypothesize that a higher

amount of radical species, which oxidized the thiolic residues, was present in samples stored in PLA packages, and this behaviour is possibly linked with the oxygen ingress occurring during the storage. The oxidation also depends on the presence of prooxidants in the product or other molecules able to activate molecular oxygen (Rowe et al., 2004; Hawkis & Davies, 2001).

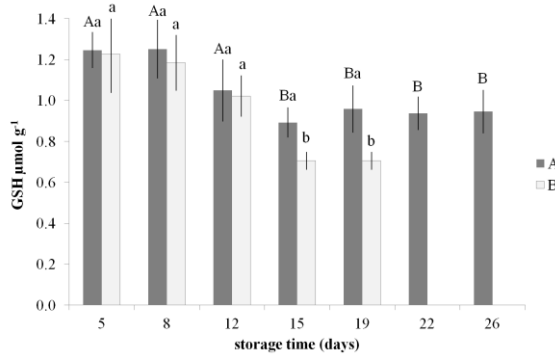


Figure 7.9. GSH content of horse meat slices placed into standard (A) and PLA packages (B), stored until 26 and 19 days respectively. ^{a-b}Different letters indicate a significant difference between samples stored in different tray. ^{A-B}Different letters indicate a significant difference between standard samples at each storage time and time 0 samples.

7.3.2 Part 2

The second part of the work focused on the evaluation of different packaging systems for fresh horse meat slices packed in low oxygen modified atmosphere, in order to evaluate the possibility to:

- substitute standard barrier materials using bio-based materials coupled with oxygen scavengers (Packaging solution A);
- compensate the lack of gas barrier capacity of bio-based materials using a master bag solution with or without oxygen scavengers (Packaging solution B);
- enhance the oxygen barrier properties of the standard solution using a master bag system (Packaging solution D).

7.3.2.1 Packaging solution A

In the first part of the work, this packaging solution was already investigated, but the combination of packaging materials and selected oxygen scavengers did not result in the preservation of meat quality. Hence, a different lid film with a higher oxygen barrier and an oxygen scavenger with higher capacity and absorption rate were used. Two different versions of the latter were considered, differing one from each other by oxygen permeability of the sachet. Materials and oxygen scavengers combination are reported in paragraph 3.4.2.2.

Headspace analyses were carried out after 0, 1, 5 and 11 days of storage (Figure 7.10). A1 solution (containing OS1, the sachet with higher gas permeability) showed the best performances in terms of oxygen scavenging, maintaining the gas level below 1% until the end of the test, while A2 (OS2) and A3 (no scavenger) showed a continuous increase of the gas concentration. The A2 samples registered a higher increase in the O₂ concentration compared to A1 samples, probably due to the different scavenger used. In fact, OS1 is characterized by a package less resistant to water and

product drips, but more permeable to oxygen than OS2; thus, the latter is not able to absorb the oxygen residual in the package neither the gas permeating through the bio-bas materials.

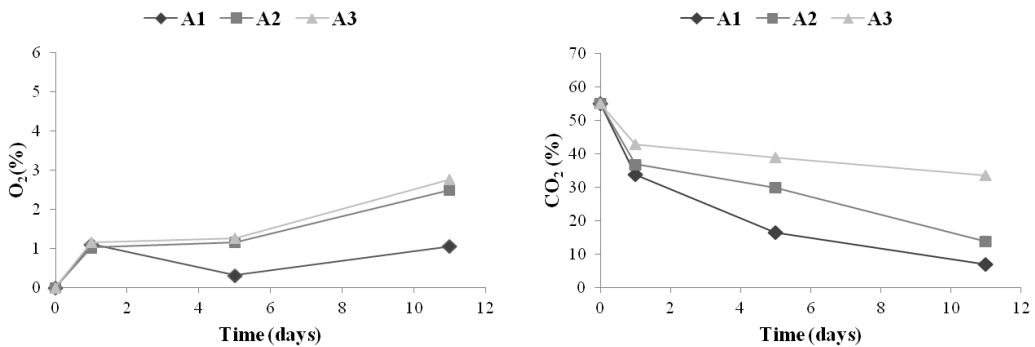


Figure 7.10. Oxygen (left) and carbon dioxide (CO₂, right) evolution in the headspace of Packaging solution A combinations during the storage.

Also if OS1 absorbed more oxygen than OS2, its activity was not enough to obtain and maintain an anoxic environment inside primary packages, which is a requirement for the preservation of horse meat quality. Also if the scavenger capacity could potentially absorb an oxygen amount equal to the sum of the oxygen residual after packaging and the gas permeating during the storage, it was not able to balance the low barrier properties of the bio-based materials. Hence, some oxygen was available to the product for the onset of oxidative reactions that degrade the meat. Furthermore, the poor gas barrier of the primary packages caused the loss of a great amount of CO₂ during the storage, hence the bacteriostatic effect of this gas could not be guaranteed.

Index	Packaging solution	Storage time (days)			
		0*	1	5	11
L*	A1		32.8±0.8 ^a	31.7±0.7 ^a	31.6±0.8 ^a
	A2	33.0±0.9	35.6±2.5 ^b	33.8±1.2 ^b	31.3±1.0 ^a
	A3		35.3±0.6 ^b	34.9±1.6 ^b	33.2±1.6 ^b
Hue angle	A1		17.7±0.9 ^a	19.8±0.2 ^a	25.5±2.0 ^b
	A2	15.4±1.3	18.7±2.5 ^a	26.0±1.5 ^b	24.2±1.5 ^a
	A3		25.5±4.3 ^b	42.0±5.5 ^c	35.2±0.8 ^c
Chroma	A1		18.2±1.1 ^a	14.5±0.3 ^a	12.5±0.9 ^a
	A2	28.4±0.9	19.1±2.1 ^a	17.1±0.8 ^b	14.0±0.4 ^b
	A3		25.0±1.9 ^b	12.5±0.5 ^c	11.6±1.0 ^c

*time 0 sample is the same for the three packaging solutions.

Table 7.2. Evolution of L*, Hue angle and Chroma of samples packaged with Packaging solution A combinations during the storage. ^{a-b}For each index, within each storage time, means with different superscript differ significantly (p<0.05).

The absence of a strictly anaerobic environment reflected into the evolution of colorimetric indexes (Table 7.2). Hue angle increased throughout the storage, moving from purple red to brown red hues: this is due to the presence of some residual oxygen that caused the irreversible oxidation of surface myoglobin, forming MetMyoglobin (MMb). The discoloration is less evident on samples A1, which packages contained an oxygen scavenger and where the concentration of oxygen was lower than 1%. The scavenging activity of the active device was not enough to prevent the oxidative phenomena, but the colour changes were not as strong as for the other samples. In the same way, Chroma values decreased from day 0 to day 11 for all samples, more evidently in the case of horse meat slices packaged in bio-based packages without any oxygen scavenger.

For a visual evaluation of the products, the digitalized images acquired at each time of analysis are reported here below (Figure 7.11). It is evident the turn of meat colour towards brown red hues already after 1 day of storage, caused by the inefficacy of all packaging solutions in preventing the ingress of oxygen inside the package.

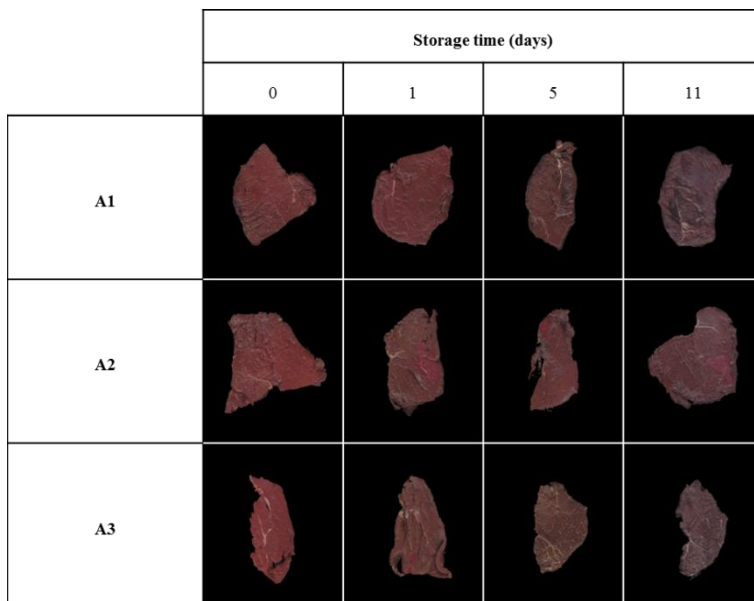


Figure 7.11. Digitalized images of samples packaged with Packaging solution A combinations during the storage.

7.3.2.2 Packaging solution B

Since the use of oxygen scavengers inside non barrier trays resulted in the failure of the test, the compensation of the barrier properties of the primary packages was pursued through the use of a master bag system. Bio-based non barrier primary packages were inserted in barrier master bags containing the same modified atmosphere, with or without oxygen scavengers inside the master bag itself and/or the trays.

The following combinations were considered:

- B3: OS2 inside the tray and OS3 inside the master bag;
- B4: OS2 inside the tray and no scavengers inside the master bag;
- B5: no scavenger inside the trays and OS3 inside the master bag;
- B6: no scavengers inside the trays neither the master bag.

Headspace analyses were carried out both on master bags and trays, in order to monitor the effect of the different combinations on the gas concentration changes. As evident in Figure 7.12, the presence of oxygen scavengers inside the master bag (B3 and B5) guaranteed very low oxygen content in trays and master bags throughout the whole storage time. The absence of the active device in solutions B4 and B6, was correlated to a higher oxygen concentration inside the master bags, thus enabling the maintenance of an anoxic environment also in the trays. In fact, when OS3 was not inserted inside the master bag, the presence/absence of the scavenger inside the tray had no influence on the headspace composition of the trays.

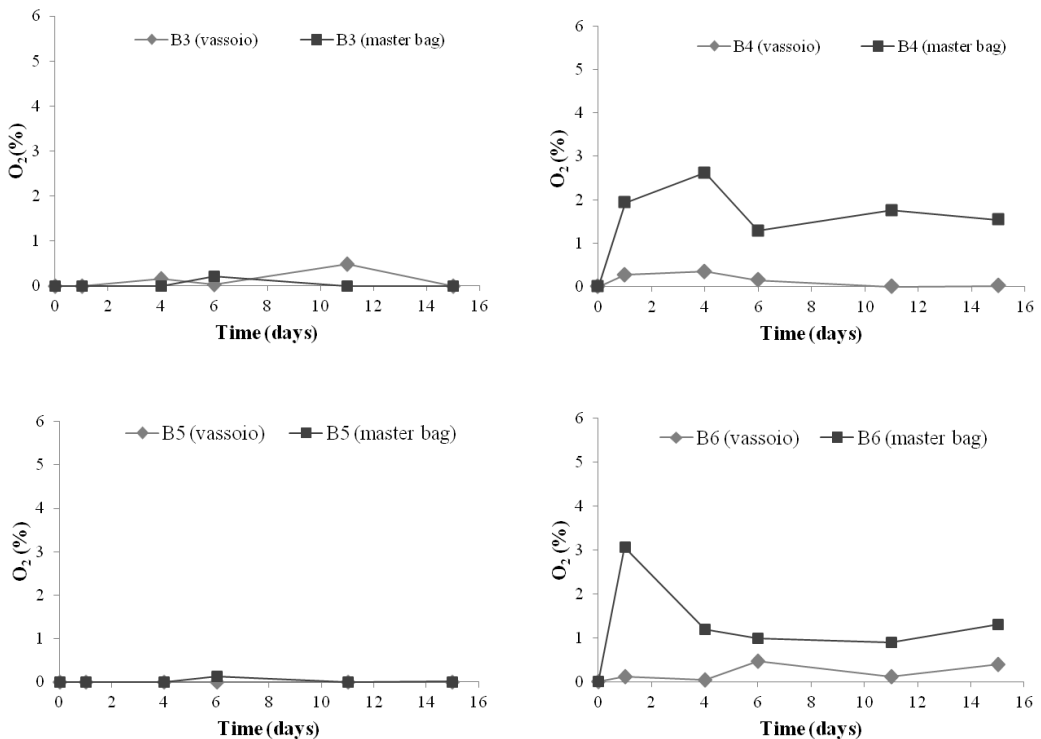


Figure 7.12. Oxygen evolution inside trays and master bags of each packaging solution B combination.

All trays tested presented an oxygen content below 1% until day 15, indicating that the sole use of the master bag containing the modified atmosphere helped in enhancing the oxygen barrier of the bio-based packages.

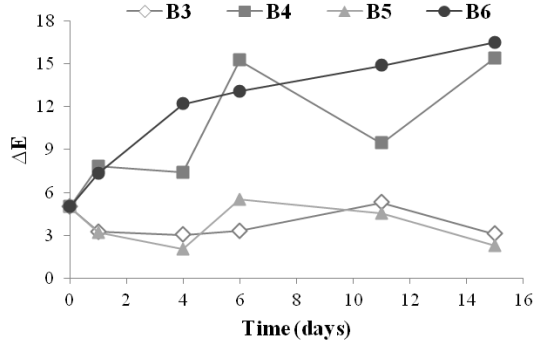


Figure 7.13. ΔE evolution of samples packed in Packaging solution B combinations.

The effects of the atmosphere composition and modification during the storage were evaluated through the colorimetric analysis and the calculation of colorimetric indexes. The main discriminating parameter is the difference between the visual appearance of samples packed in Packaging solution B combination and the control samples, packaged in standard trays containing MA. This difference is well described by the ΔE index, which calculation takes into consideration the three coordinates of the $L^*a^*b^*$ color space. Values of $\Delta E > 3$ characterize samples visibly different also from a non-trained eye; for $1 < \Delta E < 3$ differences are not evident to every eye; when $\Delta E < 1$ no differences can be detected by human eyes (Robertson, 1990).

Index	Pack. solution	Storage time (days)					
		0*	1	4	6	11	15
L*	B3		33.6±0.4 ^a	33.4±1.0 ^c	35.5±3.0 ^c	36.0±0.3 ^c	32,3±0,2 ^b
	B4	33.0±1.0	32.8±0.9 ^a	32.6±1.3 ^{ab}	31.6±0.9 ^a	30.4±0.8 ^a	29.9±0.9 ^a
	B5		32.8±0.4 ^a	31.9±0.5 ^a	34.0±1.2 ^b	32.2±0.4 ^b	33.2±0.6 ^c
	B6		33.3±1.7 ^a	33.1±0.7 ^{bc}	31.5±1.1 ^a	30.9±0.9 ^a	29.7±0.4 ^a
Hue angle	B3		13.9±0.6 ^a	13.8±2.6 ^b	12.0±3.0 ^a	17.8±0.5 ^b	11.9±1.3 ^a
	B4	15.4±1.3	19.4±5.0 ^b	16.1±2.5 ^b	30.4±0.1 ^c	16.2±2.3 ^b	21.5±5.1 ^b
	B5		13.7±0.8 ^a	11.2±1.2 ^a	18.7±1.0 ^b	11.5±0.7 ^a	10.4±0.6 ^a
	B6		18.1±1.8 ^b	27.0±4.1 ^c	27.2±6.5 ^c	28.6±3.7 ^c	22.0±5.5 ^b
Chroma	B3		25.1±0.2 ^c	23.1±1.7 ^c	24.9±1.1 ^d	23.2±0.6 ^d	24.4±0.4 ^b
	B4	28.4±0.9	18.2±0.6 ^a	18.7±0.9 ^b	11.8±0.7 ^a	16.9±3.0 ^b	12.1±3.0 ^a
	B5		22.8±0.8 ^b	24.6±0.9 ^d	21.5±0.7 ^c	20.1±1.1 ^c	25.6±1.1 ^b
	B6		19.0±1.8 ^a	147.8±2.2 ^a	14.0±0.6 ^b	10.6±0.8 ^a	11.1±1.6 ^a

*time 0 sample is the same for the 4 packaging solutions.

Table 7.3. Evolution of L^* , Hue angle and Chroma of samples packaged with Packaging solution B combinations during the storage. ^{a-b}For each index, within each storage time, means with different superscript differ significantly ($p < 0.05$).

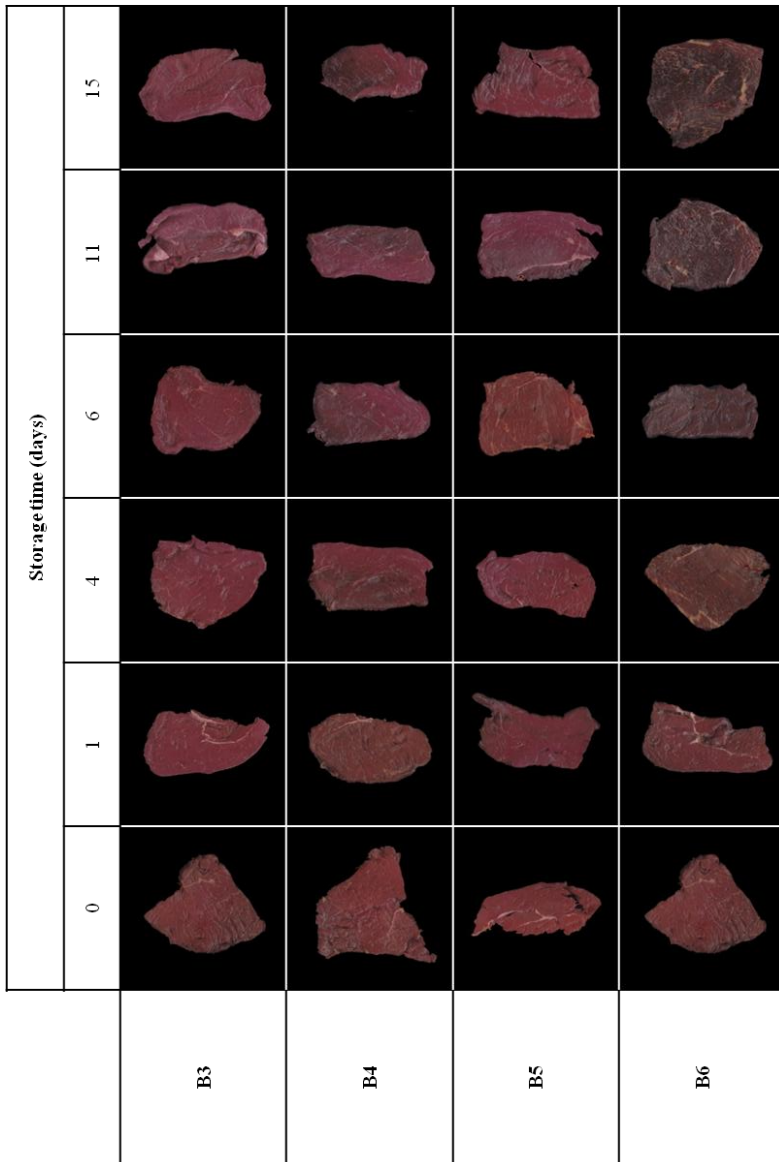


Figure 7.14. Digitalized images of samples packaged with Packaging solution B combinations during the storage.

As evident in Figure 7.13, samples can be subdivided into two groups: B3 and B5 showed similar behaviour, as well as B4 and B6. The former, which had the oxygen scavengers inside the master bag, registered a ΔE around 3 constant throughout the whole storage, hence showing a visible difference in comparison to control sample. However, since the same value was recorded at time 0, it is possible to say that that difference is correlated only to the intrinsic characteristics of the product due to the use of different batches, and that the packaging system did not influence any change in meat appearance. Differently, samples B4 and B6 showed ΔE values greater than 3 and also increasing with storage time: horse meat contained in these packages evidently changed its appearance, due to the presence of residual oxygen and the consequent oxidation phenomena.

As regarding L^* , Hue angle and Chroma indexes (Table 7.3), the same classification can be done: B3 and B5 samples maintained values almost constant during the storage, and closer to the control; while samples B4 and B6 underwent greater changes with increasing storage time, because of the oxidation of the product.

Moreover, samples B3 and B5 showed no statistical differences ($p < 0.05$) after 15 days of storage, even though only the former presented an oxygen scavenger inside the tray. Hence, the presence of the active device inside the tray is not so relevant if an oxygen scavenger is present at least inside the master bag.

Visual differences between samples stored in master bag with or without scavengers, independently from the presence of active devices inside the trays, are evident in Figure 7.14. Until 15 days of storage, samples stored in packaging solutions B3 and B5 maintained their red and homogeneous colour similar to time zero. On the contrary, samples stored in B4 and B6 master bags underwent irreversible discoloration, with brown hues appearing after few days of storage and increasing with time.

7.3.2.3 Packaging solution D

The possibility to improve the shelf life of meat stored into standard packages, already very low permeable to oxygen, was investigated in the last part of this work. Two standard packages containing horse meat slices and low oxygen modified atmosphere were inserted inside barrier master bags containing the same MA.

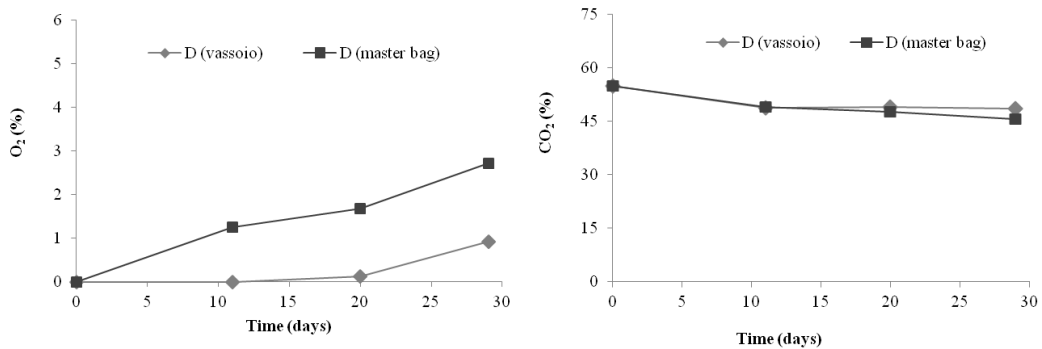


Figure 7.15. Oxygen (left) and carbon dioxide (right) evolution inside trays and master bags of packaging solution D during 29 days of storage.

Headspace composition was monitored inside both trays and master bags (Figure 7.15). Oxygen increased linearly inside master bags to a maximum of around 3% after 29 days of storage; the residual oxygen and the gas permeating through the master bag were not removed by active devices and after 20 days caused an increase of oxygen also inside the trays. In the latter, the oxygen concentration reached around 1% at day 29. As regarding carbon dioxide, after an initial loss of about 5%, likely due to its dissolution inside the product, master bags and trays headspaces were in equilibrium for the first 10 days, then master bags registered further losses reaching a final value around 45%.

Variables	Time (days)			
	0	11	20	29
ΔE	3.8±0.3	2.9±0.2	3.2±0.4	3.0±0.2
Lightness	33.0±0.9	32.1±1.0	31.8±0.5	32.6±0.9
Hue angle	15.4±1.3	11.1±2.0	11.9±1.7	13.4±2.3
Chroma	28.4±0.9	23.9±0.6	23.8±1.9	23.8±0.9

Table 7.16. Evolution of L*, Hue angle and Chroma of samples packaged with Packaging solution B combinations during the storage. ^{a-b}For each index, within each storage time, means with different superscript differ significantly (p<0.05).

Colorimetric evaluations led to the results presented in Table 7.16. ΔE index was calculated comparing samples stored using Packaging solution D and samples stored in the standard barrier packages, without oxygen scavengers and master bag. The variation of meat appearance during the storage in master bags was almost null, also if ΔE was higher than 1. In fact, it started from a high value and remained constant until days 29. As reported previously, the difference between the samples can be likely attributed only to the different meat batches analysed. In the same way, also the Lightness, Hue angle and Chroma did not vary throughout the whole storage, as a confirmation that no one of the main colour parameters was influenced by the prolonged storage.

The acceptable and constant appearance of the meat stored in master bags for 29 days can be observed in Figure 7.16.

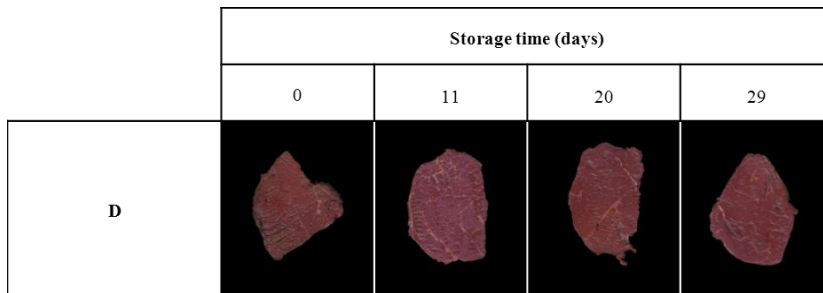


Figure 7.16. Digitalized images of samples packaged with Packaging solution D combinations during the storage.

7.4 Conclusions

The oxygen permeability of primary packages is the key factor for the preservation of horse meat quality packaged in MAP: barrier properties not so adequate to prevent and delay oxidative phenomena result in the fast degradation of meat, especially in terms of pigment oxidation. The lack of barrier properties of bio-based materials used in this work avoids their use for horse meat preservation, if not coupled with a master bag system and oxygen scavengers. Furthermore,

specific combination gave acceptable results, in particular, the insertion of oxygen scavengers inside the master bag, despite the presence or absence of additional active devices in the tray itself.

In addition, the shelf life guaranteed by the standard barrier solution (21 days) can be improved until 29 days, thanks to the presence of a gas reserve (i.e. MA inside the master bag), which reduces the possibility for oxygen to penetrate inside the trays.

In the future, we aim to deepen chemical, biochemical and nutritional properties of horse meat stored in low oxygen environments obtained thanks to the mentioned packaging system, taking into consideration for example protein pattern changes and susceptibility to digestive enzymes during storage and display life.

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Appendix 1
Conferences' abstracts

SLIM 2010, 4th Shelf Life International Meeting **Zaragoza, Spain, 23-25 June 2010**

Autori: Stefano Santagostino*, Erika Mascheroni**, Laura Franzetti**, Alessandro Adobati**,
Eleonora Uboldi**, Luciano Piergiovanni**, Sara Limbo**

*Multisorb Technologies Inc, 325 Harlem Road, Buffalo, New York 14224 USA

**DiSTAM - Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Università degli Studi di
Milano, via Celoria 2, 20133 Milano

Shelf life study of case-ready meat in a low oxygen system by means of oxygen scavengers and modified atmosphere packaging

In this study, oxygen scavengers have been evaluated in order to establish the right atmosphere conditions inside the packages, and to verify the benefits of this new technology. Standard primary packages of PVC stretch film, closed in 30% CO₂ and 70% N₂, containing about 200 g of steaks of “Eye of round” (*Semitendinosus* muscle) from pure Italian breed “*Piemontese*” were put into master barrier bags with oxygen scavengers. The samples were then stored at 1 ± 0.5 °C for 21 days in the dark. After 1, 2 and 3 weeks of storage the master bags were opened and the primary packages were exposed to air and light, measuring the time for the blooming of the meat. Myoglobin forms, color parameters of the stored and bloomed meat, microbiological tests, changes in the atmosphere composition were the key indicators monitored during the storage. Results show that with this new packaging system both retailers and final consumers could have tangible advantages such as the extension of meat distribution life and the improvement of meat quality.

10° CISETA
Rho, Italy, 10-11 maggio 2011

Autori: Eleonora Uboldi*, Alessandro Adobati*, Stefano Santagostino**, Thomas H. Powers**,
Sara Limbo*

*DiSTAM - Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Università degli Studi di
Milano, via Celoria 2, 20133 Milano

** Multisorb Technologies Food & Beverage, 325 Harlem Road, Buffalo, New York 14224 USA

**Prolungamento della vita distributiva di carne fresca bovina porzionata:
studio dell'efficacia di un sistema di confezionamento in *masterbag* in
presenza di assorbitori di ossigeno e CO₂**

Il presente lavoro si inserisce nell'ambito dello studio di nuove tipologie di confezionamento che mirano al prolungamento della vita distributiva della carne fresca, offrendo ai punti vendita della GDO maggiore flessibilità nelle produzioni. La soluzione di confezionamento proposta prevede l'inserimento di più unità di vendita (vassoi contenenti la carne già porzionata e avvolti in film estensibile) all'interno di sacchi madre barriera (*masterbags*) chiusi ermeticamente in atmosfera protettiva in presenza di assorbitori di ossigeno. Tale sistema di confezionamento è stato applicato a due tipologie di carne: carne bovina di razza Piemontese sottoposta a frollatura convenzionale e hamburger di carne macinata. I *masterbags* sono stati conservati in camera fredda a $1\pm 0,5^{\circ}\text{C}$ al buio e in seguito aperti a definiti intervalli: i vassoi, dopo una breve fase di *blooming* (riossigenazione del prodotto con ritorno al colore rosso), sono stati quindi posizionati in display refrigerato al fine di simularne la *shelf-life*. L'efficacia del sistema di confezionamento è stata valutata monitorando gli indici microbiologici, l'evoluzione delle forme mioglobiniche e del colore. Il sistema di confezionamento ha dimostrato di poter garantire il prolungamento della vita distributiva di carne fresca bovina porzionata, mantenendone qualità e sicurezza iniziali, a condizione che sia assicurata la rimozione completa dell'ossigeno residuo dall'atmosfera interna, ottenuta grazie all'uso di assorbitori di ossigeno inseriti nei *masterbags*.

**Giornata Tecnologica “La ricerca italiana sugli imballaggi attivi,
con particolare focus all'utilizzo dei nano materiali”
*Rho, Italy, 9 maggio 2011***

Autori: Eleonora Uboldi, Luciano Piergiovanni, Sara Limbo

DeFENS, Università degli Studi di Milano, via Celoria 2, 20133 Milano

Use of oxygen scavengers for fresh meat packaging

Active packaging is considered to be an innovative solution for meat packaging, overall in the case of case-ready product. A master bag packaging system, combining the most traditional tray wrapping solution with the use of oxygen scavengers as active devices, was proved to be effective for fresh meat preservation. This system is able to prolong the distribution life of fresh beef meat, sliced or ground and formed in patties, maintaining its quality for longer periods. The advantages of this packaging solutions consist in the possibility to store case-ready primary packages inside master bag with oxygen scavengers for 21 days in the case of sliced beef, and for 10 days in the case of beef patties: in both cases, after the removal from master bags, the meat presents a shelf life comparable to the traditional packed one.

The key of the success of the packaging system is the presence of oxygen scavengers, that allow the establishment and the maintenance of an anoxic environment useful for meat colour and quality preservation.

SLIM 2012, 5th Shelf Life International Meeting

Changwon, South Korea, May 30 – June 1, 2012

Autori: Eleonora Uboldi*, Aristodemo Carpen**, Stefania Iametti**, Francesco Bonomi**, Sara Limbo*

*DiSTAM, Dipartimento di Scienze e Tecnologie Alimentari, Università degli Studi di Milano, via Celoria 2, 20133 Milano, Italy

**DiSMA, Dipartimento di Scienze Molecolari Agroalimentari, Università degli Studi di Milano, via Celoria 2, 20133 Milano, Italy

Biochemical changes during anoxic storage of beef patties

Low oxygen packaging system is a common solution for the improvement of beef meat commercial life. One solution consists in packaging the meat (already prepared in the final cut) in non-barrier trays overwrapped with high permeable film, then enclose it in a master bag barrier film that contains multiple packages and an anoxic atmosphere. When a retailer needs product to stock the shelf, he could open the masterbag and remove the packages from the bag, allowing the blooming of the product for reaching the desirable, bright, cherry-red color as function of the oxygen permeability of package.

The aim of this work is to understand the main biochemical changes that occur during the anoxic storage of meat, using patties of ground beef as a case study. In particular, two main phenomena are taken into account: the *transient discoloration* during the storage in masterbag and the *blooming*. During the storage in master bag, the anoxic environment created by the oxygen scavengers allowed myoglobin deoxygenation through the formation of the reversible MetMyoglobin (MMb). This phenomenon (*transient discoloration*) took few days: after this short period the reversion of MMb was complete and, once the masterbag was opened, the *blooming* occurred as the result of oxygen binding to the iron atom.

In order to follow these phenomena, patties of ground beef were primarily packed into EPS trays wrapped with a PVC stretch film, then inserted into a barrier master bag (nylon based) with two ready to use oxygen scavengers (OS). Masterbags were flushed with the anoxic atmosphere (30% CO₂, 70% N₂) and then stored in the dark at 1±0.5°C for 4, 7 and 10 days; after master bag opening, the trays were stored in the dark at 3±1°C for the blooming time and finally placed into a display case at 5±1°C for simulated display life. Meat changes were evaluated following surface myoglobin, colour evolution, proteolytic events and lipid oxidation. Also, glutathione content (GSH) was determined in order to evaluate its involvement in transient discoloration mechanism and cytochrome c release to verify the mitochondrial respiratory activity.

In fact, antioxidant enzymes, such as glutathione that is largely present in meat products, are supposed to be the mayor actors in the maintenance of ferrous “free” iron and redox potential of the meat. Results confirmed that GSH pool is not depleted during the first days of the anoxic storage, when the *transient discoloration* occurs, indicating its possible role in maintaining the ferrous form myoglobin. The cytochrome c release was a good indicator of the respiratory activity: in fact, the results highlighted that blooming was faster and more pronounced in patties aged for longer periods, owing to some loss of activity of the oxygen-consuming enzymes, and a deeper OxyMb layer was formed. In fact, the depth to which the oxygen penetrates depends on a balance between

oxygen concentration at the surface and tissue respiration, which consumes oxygen as it becomes available.

All these evaluations could be helpful to explain meat changes during and after the storage in low oxygen master bag, in order to better comprehend these two visual phenomena from a biochemical perspective.

SLIM 2012, 5th Shelf Life International Meeting

Changwon, South Korea, May 30 – June 1, 2012

Autori: Eleonora Uboldi*, Stefano Santagostino**, Thomas H. Powers**, Laura Franzetti*, Luciano Piergiovanni*, Sara Limbo*

*DiSTAM, Dipartimento di Scienze e Tecnologie Alimentari, Università degli Studi di Milano, via Celoria 2, 20133 Milano, Italy

** Multisorb Technologies Food & Beverage, 325 Harlem Road, Buffalo, New York 14224 USA

Effects of anoxia on meat storage: the case of beef patties packaged in a master bag system with oxygen scavengers

The meat packaging scenario offers a wide range of solutions, from traditional packaging to highly innovative systems that are meant to increase meat commercial life.

Master bag packaging of multiple display-ready beef combined with low oxygen modified atmospheres represents a solution with high potentialities, both in terms of the extension of distribution life and the reduction of losses at retail level.

The aim of this work was to evaluate the effectiveness of this kind of packaging solution in prolonging the distribution life of patties made by ground beef meat, taking into consideration safety and quality issues as a function of the performances of the whole system.

The master bag packaging system consisted of overwrapped permeable trays containing the patties (primary packaging), inserted into a gas barrier master bag with modified atmosphere and O₂ scavengers, used to achieve the right oxygen residual level. In fact, very low oxygen concentration have to be reached to maintain myoglobin in a deoxygenated state and to allow the substantially complete reoxygenation of the meat surface when the trays are removed from the master bag, prior to be displayed. In particular, two trays were inserted into a gas barrier master bag (nylon based) with two ready to use oxygen scavengers (FreshPax CR®). Master bags were flushed with the atmosphere (30% CO₂, 70% N₂) and then stored in the dark at 1±0.5°C for 4, 7 and 10 days; after the master bag opening, the trays were stored in the dark at 3±1°C for the blooming time and finally placed into a display case at 5±1°C to simulate the display life. Meat changes were evaluated during the three main steps (*storage in the master bag*, *blooming* and *display life*) following microbiological indexes, surface myoglobin forms, visual acceptance, colour evolution, lipid oxidation and proteolytic events. In this study, the performances of the system were also optimized taking into consideration the kinetics of the oxygen scavenger and the role of oxygen transmission rate of the tray and the wrap film on patties quality. In a parallel trial, a challenge microbial test (CMT) was carried out on patties inoculating *Listeria monocytogenes* strains before their packaging inside the master bags. The aim was to determine the potential of growth of this microorganism and, more specifically, to understand if the anoxic conditions can or cannot support its growth during the storage and the display life.

The evolution of all the selected safety and quality indexes confirms the effectiveness of this packaging system. Also, it is possible to affirm that this packaging system allows a better management of the meat products chain, reducing losses and wastes through the extension of the storage life.

XVII Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology *Cesena 19-21 September, 2012*

Autori: Eleonora Uboldi^a

^aDeFENS, Università degli Studi di Milano, via Celoria 2, 20133 Milano, Italy.

Effects of Anoxic Storage on Beef Patties Quality

The aim of this PhD project is to study the effect of anoxia on meat products, in particular the application of a master bag packaging system to red meat, using a low-oxygen modified atmosphere and oxygen scavengers, for the improvement of meat commercial life. Case-ready beef patties were studied, following the evolution of sensory, chemical, biochemical and microbiological indexes, in order to prove the efficiency and efficacy of the system and to ensure the maintenance of meat quality during and after the anoxic storage.

Effetti del confezionamento in anossia sulla qualità di hamburger di carne rossa

Il progetto di dottorato ha come scopo lo studio degli effetti dell'anossia sui prodotti carnei ed in particolare l'utilizzo di un sistema di confezionamento in master bag, contenenti atmosfera modificata a basso contenuto di ossigeno e assorbitori di ossigeno, per il prolungamento della vita distributiva di carne rossa. Sono stati studiati hamburger di carne bovina case-ready seguendo l'evoluzione di indici sensoriali, chimici, biochimici e microbiologici, al fine di provare l'efficacia del sistema e il mantenimento della qualità della carne così conservata.

Key words: Beef meat, low oxygen MAP, master bag, cytochrome *c* release, glutathione.

**2012 CIGR Section VII International Technical
Symposium on “*Innovating the Food Value Chain*” -
Postharvest Technology and Agri-Food Processing
Stellenbosch, South Africa, 25-29 November, 2012**

Autori: Eleonora Uboldi^a, Mirka Lamperti^a, Stefania Iametti^a, Thomas H. Powers^b, Sara Limbo^a

^aDeFENS, Università degli Studi di Milano, via Celoria 2, 20133 Milano, Italy.

^bMultisorb Technologies, 325 Harlem Road, Buffalo, NY, 14224, USA

Master bag low-oxygen packaging system effects on case-ready beef meat

Red meat is one of the most degradable food products, so generally meat packaging systems are focused on the prolongation of its distribution or display life. A master bag packaging system, coupled with low-oxygen modified atmosphere generated by the use of oxygen scavengers, was studied in order to improve beef meat distribution life. Case-ready units of sliced beef meat and beef patties were packed in master bags, stored for few days or weeks in refrigerated conditions, then exposed to air for the re-oxygenation (blooming phase) and finally exposed on a refrigerated display case in order to simulate the retail display life. Microbiological analyses, myoglobin forms estimation, colour indexes, iron oxidative state, reduced glutathione and cytochrome c release were monitored as main quality indexes. In both cases, the main phenomenon observed was the transient discoloration of myoglobin on meat surface during the first days of anoxic storage, but at each opening time of master bags the meat was able to bloom fully on the upper side, ensuring good performances during the subsequent display life. Concerning biochemical indexes in patties, a slight reduction of GSH content and a small increase of released cytochrome c were observed at longer storage time. That indicates an increasing stress inside the product, probably correlated with meat ageing in anoxic conditions, also if the meat is still safe and has a good quality considering all the other parameters. In general, it is possible to state that this system could extend meat distribution life of beef meat (sliced or ground), ensuring also the maintenance of the good quality of the product and improving the sustainability of the distribution chain through the reduction of food wastes.

**2012 CIGR Section VII International Technical
Symposium on “*Innovating the Food Value Chain*” -
Postharvest Technology and Agri-Food Processing
Stellenbosch, South Africa, 25-29 November, 2012**

Autori: Eleonora Uboldi^a, Thomas H. Powers^b, Joseph H. Hotchkiss^c, Sara Limbo^a

^aDeFENS, Università degli Studi di Milano, via Celoria 2, 20133 Milano, Italy.

^bMultisorb Technologies, 325 Harlem Road, Buffalo, NY, 14224, USA

^cSchool of Packaging, Michigan State University, 130 Packaging Building East Lansing, MI, 48824, USA

**Active packaging and low-oxygen MAP as tools for fresh and processed meat
quality preservation**

Modified atmosphere (MA) and active packaging are generally recognized as useful tools in the maintenance of food quality, assuring a longer shelf life, a better appearance, and a greater acceptance of the products. The aim of this study was to investigate the effects of MA and active packaging (i.e. oxygen absorbers) combinations in the prolongation of fresh and processed meat commercial life, through the inhibition of microbial spoilage and colour degradation. In the case of sliced cooked ham, the product was packed using films with moderately low OTR, oxygen scavengers and eventually flushed with MA (78% CO₂, 2.4% O₂, bal. N₂). Also if a high barrier was not provided by the packaging material, oxygen absorbers retarded the growth of psychrotrophic bacteria, yeasts, and moulds and preserved colour as measured by L values of sliced ham, especially in CO₂ flushed high barrier packages, throughout 79 days. Fresh meat was packed in single case-ready units, inserted into a barrier master bag with oxygen scavengers and then flushed with MA (30% CO₂, 70% N₂). Its commercial life was prolonged to 21 days, ensuring also a subsequent shelf life of 48 hours, as regarding microbial spoilage and colour parameters (Hue angle, Chroma). Prolonging the commercial life of fresh and processed meat products, thanks to MA and active packaging combination, may contribute to the reduction of food losses and wastes in the whole supply chain.

**5th International Symposium on Food Packaging - Scientific
Developments supporting Safety and Innovation
ILSI Europe
Berlin, Germany, 14-16 November 2012**

Autori: E. Uboldi*, A. Adobati*, L. Piergiovanni*, T.H. Powers**, S. Santagostino**, S. Limbo*

*DeFENS – Department of Food, Environmental and Nutritional Sciences, Università degli Studi di Milano,
via Celoria 2, 20133 Milano, Italy

** Multisorb Technologies, 325 Harlem Road, Buffalo, New York – 14224 USA

**The role of oxygen scavengers in anoxic storage of beef meat: the case of
patties packed in a master bag system**

ABREVIATIONS: MA modified atmosphere, EPS expanded polystyrene, EVOH ethylene vinyl alcohol, PVC polyvinylchloride.

BRIEF INTRODUCTION

Ultra-low oxygen atmospheres with carbon dioxide and nitrogen can be used to preserve the quality of beef meat. The limit of this kind of packaging is that very low oxygen concentration is required to maintain myoglobin in a deoxygenated state and to allow the right oxygenation of the meat surface prior to display. Oxygen scavengers can be used to achieve the right oxygen residual level but the challenge is to adapt the scavenger effectiveness to the real packaging system and to the meat oxygen sensitivity. In this study, the role of oxygen scavengers in maintaining the safety and quality of case-ready patties of minced beef was considered. The packaging system consisted in overwrapped permeable trays containing the patties, inserted into gas barrier master bag with MA and O₂ scavengers.

METHODS AND MATERIALS

Patties of minced beef meat were primarily packed into EPS trays wrapped with a PVC stretch film (O₂TR 22000 cm³m⁻² 24h⁻¹bar⁻¹ at 23°C). Two trays were inserted into a barrier master bag (EVOH based) with two ready to use iron-based oxygen scavengers. Master bags were flushed with 30/70 CO₂/N₂. Master bags were stored in the dark at 1±0.5°C for 4, 7 and 10 days. After the opening of master bags, the trays were stored in the dark at 3±1°C for the *blooming* phase and finally placed onto a display case at 5±1°C to simulate the *display life*. Meat changes were evaluated following microbiological indexes, myoglobin forms (1) and colour evolution.

RESULTS AND CONCLUSIONS

Different typologies of iron-based scavengers were analysed in order to find the right kinetic of absorption in the adopted packaging system. The evolution of the gas inside the master bag was followed using a non-invasive device. During the storage, the anoxic environment created by the selected scavengers allowed myoglobin deoxygenation through the formation of the reversible MetMyoglobin (MMb) (2). This phenomenon (*transient discoloration*) took few days: after 7 and 10 days, the reversion of MMb was complete so, once the master bag was opened, the *blooming*

was studied in depth, following the kinetic of oxygenation both on the upper and the bottom surface of the patties. The blooming of patties packaged in master bags with O₂ scavenger for 7 and 10 days was fully and rapid, while the absence of scavengers allowed the establishment of low but critical oxygen concentration (3) that promoted the oxidation instead of the oxygenation of the meat surface. In this study, the scavenger effectiveness of the active devices was also optimized taking into consideration the role of oxygen transmission rate of the tray and the wrap film on patties quality during *storage in master bag*, *blooming* and *display life* steps.

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Autori: Eleonora Uboldi^a

^aDeFENS, Università degli Studi di Milano, via Celoria 2, 20133 Milano, Italy.

Effects of Modified Atmosphere Packaging on Red Meats Quality Preservation

The aim of this PhD project is to study the effect of anoxia on meat products, in particular the application of a master bag packaging system to red meat, using a low-oxygen modified atmosphere and oxygen scavengers, for the improvement of meat commercial life and the preservation of its nutritional value. Case-ready beef slices, ground beef patties and sliced horse meat were studied, following the evolution of sensory, chemical, biochemical and microbiological indexes, in order to evaluate the efficiency and efficacy of the system on the preservation of fresh meat quality during the anoxic and the oxygenated phases of the storage.

Effetti del confezionamento in atmosfera modificata sul mantenimento della qualità di carni rosse

Il progetto ha come scopo lo studio degli effetti dell'anossia sui prodotti carnei ed in particolare l'utilizzo di un sistema di confezionamento in master bag, contenenti atmosfera modificata a basso contenuto di ossigeno e assorbitori di ossigeno, per il prolungamento della vita distributiva di carne rossa ed il mantenimento delle sue proprietà nutrizionali. Sono state studiate soluzioni case-ready di carne bovina a fette, hamburger di carne bovina e carne equina a fette, seguendo l'evoluzione di indici sensoriali, chimici, biochimici e microbiologici, al fine di provare l'efficacia del sistema e il mantenimento della qualità della carne così conservata.

Key words: Red meat, low oxygen MAP, master bag, shelf life, blooming.

Agorà - incontro nazionale sul food packaging

Monza, 17-18 ottobre 2013

Autori: Eleonora Uboldi^a, Luciano Piergiovanni^a, Stefano Dessì^b, Emanuele Raimondi^b, Sara Limbo^a

^aUniversità degli Studi di Milano, Dipartimento di Scienze per gli Alimenti, la Nutrizione e l'Ambiente – DeFENS, Packaging Division

^bNaba Carni spa, Via Prati, 69, 25086 Rezzato (Bs), Italia

Ruolo di materiali e sistemi di condizionamento in anossia nel prolungamento della shelf-life di carne equina *case-ready*

Una richiesta sempre più frequente è quella di individuare e/o ottimizzare soluzioni per estendere la *shelf life* di prodotti freschi porzionati e serviti in unità consumatore, al fine di raggiungere nuovi mercati e rispondere a specifiche esigenze di sostenibilità, garantendo qualità e sicurezza.

Il presente lavoro ha avuto come obiettivo quello di valutare l'efficacia di diversi sistemi di conservazione per carne fresca di cavallo a fette, confezionata in unità consumatore contenenti atmosfera modificata priva di ossigeno. La carne equina infatti possiede caratteristiche nutrizionali che la differenziano dalle altre carni, soprattutto per l'elevato contenuto in ferro ed in acidi grassi polinsaturi. Queste caratteristiche rendono la carne equina molto sensibile all'ossigeno e il suo confezionamento è un processo delicato e complesso che richiede tecniche ed interventi opportuni.

Nel corso della sperimentazione sono state testate le seguenti soluzioni: *a*) vassoi in polistirene espanso saldati con film laminato, entrambi ad alta barriera ai gas, con e senza assorbitori di ossigeno (OS) internamente al vassoio; *b*) vassoi in acido polilattico espanso (XPLA) chiusi con film barrierato in PLA, posizionati all'interno di *master bag* barriera; *c*) soluzione *case ready* descritta al punto *b* posizionata all'interno di *master bag*, con e senza assorbitori di ossigeno nello spazio di testa del sacco.

Per la valutazione delle *performances* del sistema di confezionamento e la conseguente qualità del prodotto, sono state condotte analisi della composizione gassosa dello spazio di testa (*master bag* e vassoi), analisi microbiologiche, colorimetriche e sensoriali, e acquisizione delle immagini tramite scanner. L'inserimento di più unità di vendita in sacchi barriera e la presenza di assorbitori di ossigeno all'interno dei *master bag* diventa l'elemento discriminante per il mantenimento dei parametri qualitativi di carne conservata in soluzioni a base di XPLA. I risultati ottenuti indicano infatti che gli assorbitori d'ossigeno svolgono un ruolo fondamentale nel compensare le proprietà di barriera dei materiali a base di acido polilattico quando abbinati ad un confezionamento anossico in *master bag*. In queste condizioni, la carne equina raggiunge 15 giorni di conservazione a $0.5\pm 0.5^{\circ}\text{C}$, aprendo ad uno scenario distributivo che contempli l'impiego di bio-plastiche. Nelle soluzioni in cui i materiali ad alta barriera vengono usati in combinazione ai *master bag*, la doppia protezione del prodotto riduce fino ad annullare la forza motrice che regola gli scambi gassosi tra l'esterno e l'interno del vassoio, proteggendo la carne dall'esposizione all'ossigeno e consentendo il prolungamento della conservazione fino ad almeno un mese a $0.5\pm 0.5^{\circ}\text{C}$.

In conclusione, la necessità di mantenere l'anossia per tutta la durata della conservazione, dalla distribuzione alla vendita, impone la selezione di materiali tradizionali ad alta barriera o di

materiali di nuova concezione e con minore impatto ambientale come il PLA espanso, soprattutto se soluzioni attive vengono impiegate nel controllo degli scambi gassosi.

Agorà - incontro nazionale sul food packaging

Monza, 17-18 ottobre 2013

Autori: Eleonora Uboldi^a, Miriam Zanoletti^a, Thomas H. Powers^b, Sara Limbo^a

^aUniversità degli Studi di Milano, Dipartimento di Scienze per gli Alimenti, la Nutrizione e l'Ambiente – DeFENS, Packaging Division

^bMultisorb Technologies, 325 Harlem Road, Buffalo, NY 14224

Ottimizzazione di un sistema di confezionamento in master bag per hamburger di carne bovina macinata

Il presente lavoro ha avuto lo scopo di valutare l'efficacia di un sistema di confezionamento per il prolungamento della vita commerciale di carne fresca bovina, ed in particolare hamburger di carne macinata. Il sistema consiste nell'inserimento di più unità *case-ready* (vassoi contenenti carne porzionata, avvolti in film estensibile con elevata permeabilità ai gas) all'interno di sacchi master bag barriera, chiusi ermeticamente in atmosfera modificata (CO₂ e N₂) e in presenza di assorbitori di ossigeno, capaci di mantenere condizioni anossiche all'interno del sistema. La finalità è quella di sfruttare la capacità del pigmento mioglobinico di riportarsi in forma ossigenata dopo la permanenza della carne in ambiente anossico grazie al fenomeno di *blooming*. Infatti, grazie alla permeabilità elevata del film avvolgente il vassoio la deossimioglobina superficiale si ossigena nuovamente, facendo riacquistare alla carne la sua colorazione rossa.

Durante la sperimentazione, le seguenti analisi sono state eseguite: determinazione dell'ossigeno residuo all'interno dei master bag; analisi colorimetriche (L*, a*, b*, Hue angle, Chroma); stima delle forme mioglobiniche superficiali; analisi microbiologiche (CBT, *Pseudomonas* spp., batteri lattici, *Enterobacteriaceae*); determinazione dell'ossidazione lipidica secondaria (malondialdeide); acquisizione di immagini tramite scanner.

Sono stati individuati due periodi ottimali di conservazione della carne in master bag a 0.5°C, ovvero 8 e 10 giorni, in seguito ai quali è stata poi ottimizzata la fase di *blooming*, fissando in due ore il tempo di riossigenazione superficiale. La perforazione del film estensibile (effettuata dopo l'apertura del master bag) non influenza la cinetica di *blooming* e le differenze tra campioni forati e non-forati sono risultate non statisticamente significative (p<0.05). Dopo la fase di *blooming*, i campioni sono stati posizionati in display refrigerato al fine di simulare la shelf life nel punto vendita. Durante la display life, tutti i parametri qualitativi considerati hanno registrato un'evoluzione paragonabile a quella misurata sul prodotto conservato tradizionalmente in una soluzione *tray-wrap* in aria (controllo).

In conclusione è possibile affermare che questo sistema di confezionamento in master bag è in grado di garantire il prolungamento della vita distributiva di hamburger di carne bovina macinata fino a 10 giorni in master bag, e successivi 2 giorni di display life. Quindi, il sistema si dimostra competitivo nei confronti della tradizionale soluzione *tray-wrap*.

Agorà - incontro nazionale sul food packaging **Monza, 17-18 ottobre 2013**

Autori: Eleonora Uboldi^a, Alessandro Adobati^a, Miriam Zanoletti^a, Luciano Piergiovanni^a, Sara Limbo^a

^aUniversità degli Studi di Milano, Dipartimento di Scienze per gli Alimenti, la Nutrizione e l'Ambiente – DeFENS, Packaging Division

Evoluzione della qualità di carne bovina a fette confezionata in atmosfera protettiva in combinazione con assorbitori di ossigeno

Oggetto del presente lavoro è stata la valutazione della qualità di carne bovina a fette (*Semiteminosus*) durante e in seguito alla conservazione in anossia in un sistema *master bag*. Tale sistema di confezionamento prevede l'inserimento di vassoi *case-ready* e di assorbitori di ossigeno all'interno di un *master bag* barriera poi chiuso ermeticamente in atmosfera modificata (30% CO₂, 70% N₂). La presenza degli assorbitori di ossigeno garantisce il mantenimento dell'anossia per tutta la durata della conservazione nel sacco. Tale condizione è infatti fondamentale per preservare la qualità del prodotto poiché le basse concentrazioni di ossigeno possono causare l'ossidazione irreversibile dei pigmenti compromettendo quindi l'accettabilità del prodotto. Si è voluto sottoporre alla sperimentazione lo stesso taglio bovino proveniente da due diverse razze (Piemontese e Argentina) con differente storia prima del porzionamento (rispettivamente di frollatura aerobica e sottovuoto, entrambe a 2±1°C). Durante tutte le fasi di conservazione (*storage life*, *blooming* e *display life*) sono stati valutati indici microbiologici, colorimetrici e spettrofotometrici (stima delle forme mioglobiniche superficiali), oltre all'acquisizione delle immagini tramite scanner.

Entrambe le tipologie di carne sono state conservate in *master bag* per almeno 21 giorni, durante i quali non si è riscontrato un decadimento significativo degli indici qualitativi. Questo risultato può essere anche spiegato dalla funzione sequestrante degli assorbitori: infatti, già a partire dal secondo giorno di conservazione non è stato più possibile rilevare la presenza di ossigeno all'interno dei sacchi.

In seguito all'apertura del *master bag* i pigmenti superficiali si riossigenano e conferiscono al prodotto il colore rosso acceso tipico della carne fresca. Grazie alla ripresa della colorazione è stato possibile esporre i prodotti in un display refrigerato per simularne la vita di scaffale nel punto vendita. L'esposizione nel banco frigo è stata condotta per 4 giorni durante i quali si è osservata la graduale ossidazione dei pigmenti superficiali che ha comportato il viraggio del colore verso tonalità marroni. La degradazione del prodotto durante la *display life* non è risultata favorita dal confezionamento in *master bag* rispetto a quello tradizionale (*tray wrap*). È quindi possibile concludere che il sistema di confezionamento in *master bag* garantisce un aumento della vita commerciale di differenti tipologie di carne bovina a fette fino a 21 giorni, senza compromettere l'accettabilità del prodotto.

Agorà - incontro nazionale sul food packaging **Monza, 17-18 ottobre 2013**

Autori: Eleonora Uboldi^a, Sara Limbo^a, Luciano Piergiovanni^a, Thomas H. Powers^b

^aUniversità degli Studi di Milano, Dipartimento di Scienze per gli Alimenti, la Nutrizione e l'Ambiente –
DeFENS, Packaging Division

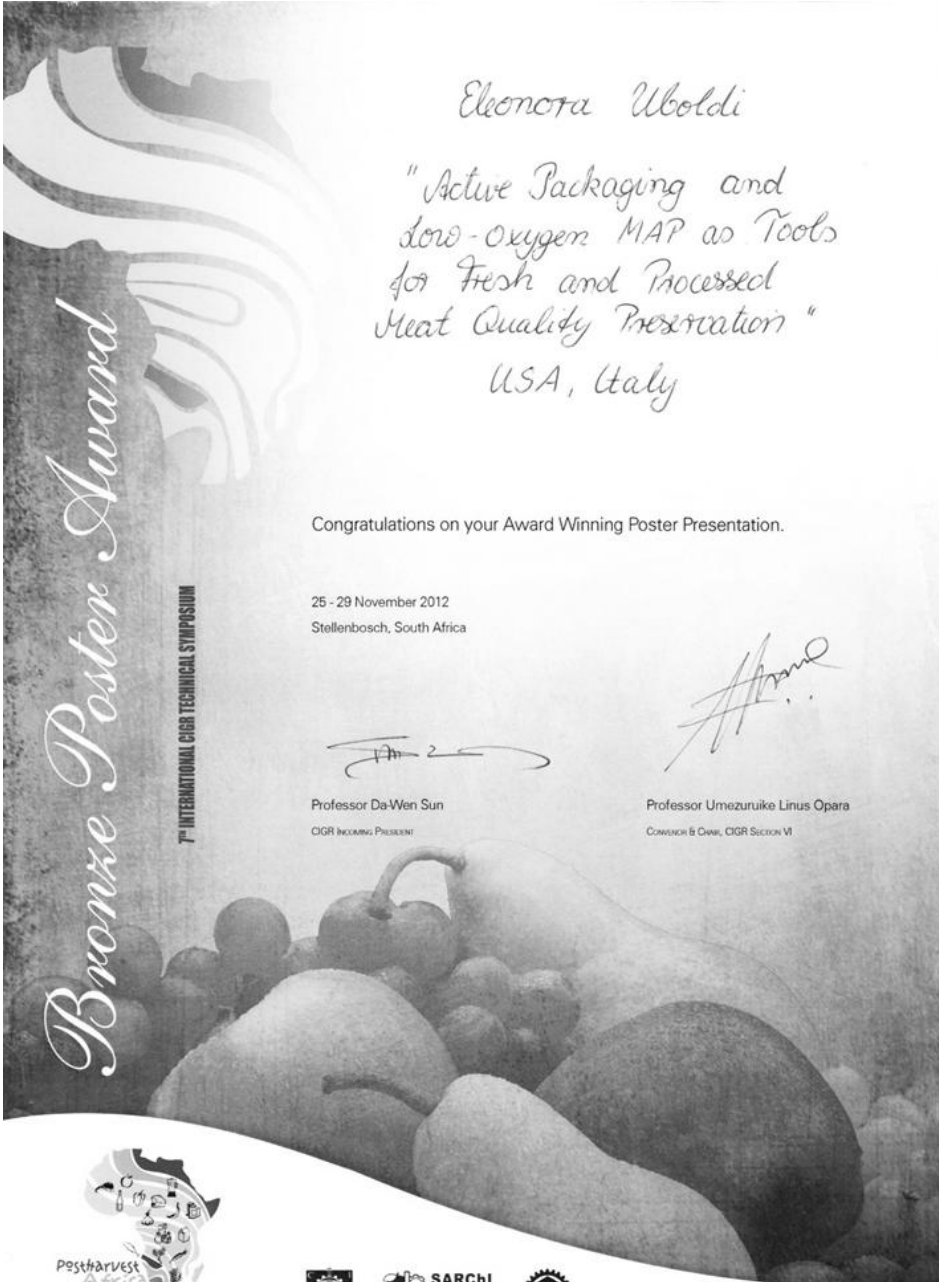
^bMultisorb Technologies, 325 Harlem Road, Buffalo, NY 14224

Prolungamento della vita distributiva di carne fresca bovina: confezionamento in master bag con assorbitori di ossigeno (MAPLOX[®])

Nel panorama dei sistemi di confezionamento per la carne fresca, un posto importante è occupato dai sistemi basati sull'uso di atmosfere modificate (o protettive). Questi ultimi sono generalmente classificati in base al contenuto di ossigeno della miscela adottata, alto o basso. La presenza di elevate concentrazioni di ossigeno (80%) all'interno della confezione permette di mantenere i pigmenti superficiali nello stato ossigenato, che conferisce al prodotto una colorazione rosso vivo, associata dal consumatore alla carne fresca, ma allo stesso tempo favorisce l'ossidazione della frazione lipidica. Al contrario, l'uso di atmosfere modificate prive di ossigeno rallenta i processi ossidativi, ma conferisce al prodotto una colorazione porpora, non sempre gradita. Per ovviare a quest'ultimo problema è possibile ricorrere a soluzioni che combinano la conservazione in anossia e quella aerobia, come ad esempio il sistema MAPLOX[®]. Tale sistema consiste nell'inserimento di confezioni primarie (*case ready*) in sacchi madre barriera, in presenza di assorbitori di ossigeno e atmosfera modificata a basso contenuto di ossigeno. Durante la conservazione in master bag, il prodotto carneo acquisisce una colorazione porpora, che ritorna però ad essere rosso acceso una volta che le confezioni sono estratte dal master bag ed esposte all'ossigeno atmosferico. Le potenzialità di tale sistema sono legate all'ottimizzazione dello stesso in funzione del prodotto carneo considerato. La confezione primaria deve presentare uno spazio di testa ridotto il più possibile, al fine di avere il minor contenuto possibile di ossigeno residuo, e i materiali utilizzati devono avere elevata permeabilità all'ossigeno. Questo consente un corretto scambio gassoso tra l'interno della confezione e l'ambiente circostante (master bag o atmosfera), alla base del meccanismo di conversione della mioglobina, la principale responsabile del colore della carne. Inoltre, durante la fase di confezionamento in master bag, è fondamentale l'impostazione della macchina confezionatrice per una corretta ed efficace evacuazione dell'aria dal sistema e l'inserimento di un'adeguata quantità di atmosfera modificata. La presenza degli assorbitori di ossigeno è necessaria per assicurare la rimozione dell'ossigeno che residua all'interno del sistema. Vanno quindi scelti in base alla capacità e alla velocità di assorbimento. Anche la selezione della miscela gassosa corretta è in funzione non solo dell'animale da cui proviene la carne, ma anche del taglio e porzionatura. Carni macinate o tagliate in piccoli pezzi sono infatti più sensibili ai fenomeni di degradazione rispetto ai prodotti a fette o a pezzo intero, e quindi i primi richiedono quantità più elevate di CO₂ al fine di garantire la salubrità del prodotto per il tempo desiderato. Non da ultimo, la qualità del prodotto all'origine è di fondamentale importanza, poiché carni con cariche microbiche particolarmente elevate vanno comunque incontro a fenomeni di degradazione rapidi.

Appendix 2
Honours and Awards

2012 CIGR Section VII International Technical Symposium on “Innovating the Food Value Chain” - Postharvest Technology and Agri-Food Processing Stellenbosch, South Africa, 25-29 November, 2012



Bronze Poster Award

7th INTERNATIONAL CIGR TECHNICAL SYMPOSIUM

Eleonora Uboldi

"Active Packaging and Low-Oxygen MAP as Tools for Fresh and Processed Meat Quality Preservation"

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Appendix 3
Publications



Shelf life of case-ready beef steaks (*Semiteminosus* muscle) stored in oxygen-depleted master bag system with oxygen scavengers and CO₂/N₂ modified atmosphere packaging

S. Limbo^{a,*}, E. Uboldi^a, A. Adobati^a, S. Iametti^a, F. Bonomi^a, E. Mascheroni^a, S. Santagostino^b, T.H. Powers^b, L. Franzetti^a, L. Piergiovanni^a

^a Department of Food, Environmental and Nutritional Sciences, Università degli Studi di Milano, Via Celoria 2, 20133 Milano, Italy

^b Multisorb Technologies, 325 Harlem Road, Buffalo, NY 14224, USA

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ABSTRACT

This study aims to evaluate the stability of beef from *Semiteminosus* muscle packaged in oxygen permeable wrapped-tray units and stored in a master bag system, with and without oxygen scavengers. Changes in the atmosphere composition, microbiological indexes, myoglobin forms and color parameters were monitored during the storage in master bag, blooming and display life. The presence of scavengers reduced rapidly the oxygen concentration and maintained it at values not detectable instrumentally. Within few days of storage in master bags, the resolution of the transient discoloration was completed and the meat quality was maintained over the anoxic storage. After the removal from master bags meat bloomed completely reaching OxyMb level and Chroma values higher than those on fresh meat at t_0 . During 48 h of display life at 4 °C, quality attributes had a decay slower than samples stored traditionally in air. Without scavengers the oxygen caused the irreversible discoloration within 7 days, due to the formation of metmyoglobin on the surface.

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1. Introduction

Meat that is prepared at retail level is usually part of boxed meat systems where primal cuts are produced and often vacuum packaged (VP) at the packing plant and distributed to the retail centers. There, the primal cuts are further processed (cut, minced, etc.) with the aim to package in consumer units that afterwards are stored in refrigerated display for few days. The most important trend that stands out year after year in case-ready meat packaging is the need to reduce labor in the back of the retail store (Belcher, 2006).

One solution consists in packaging the meat (already prepared as the final cut) in trays overwrapped with an high gas permeability film, then enclosed in a larger master bag that contains multiple packages. When a retailer needs product to stock the shelf, he opens the master bag and removes the packages from the bag. The product should bloom forming the desirable, bright, red color as function of the oxygen permeability of the film that overwraps individual trays. The absence of O₂ in low-oxygen packages usually maintains myoglobin in a reversibly reduced state (thus having a purplish-red color, unfamiliar to many consumers) and minimizes oxidative deteriorative reactions. The limit of this kind of packaging is that a very low oxygen concentration is required to maintain myoglobin in a deoxygenated state and stringent optimization of the whole packaging system is also needed. Different

information about the O₂ critical levels for meat is available in the literature. Gill (1996) underlined that an O₂ residual level at a maximum of 0.1%, but preferentially, at no more than 0.05% is recommended to guarantee the blooming of meat upon re-exposure to air. Similar results were reported by Mancini and Hunt (2005), who indicated a critical value of 0.05% O₂ (500 ppm) for beef. Tewari, Jayas, Jeremiah, and Holley (2001) distinguished a limit lower than 100 ppm for beef with poor color stability and lower than 600 ppm for beef with high color stability when stored at sub-zero temperatures. Again, for ground beef, oxygen levels lower than 10 ppm were found to optimize red color stability of beef during retail display (Gill & McGinnis, 1995). In fact, a critical point is that, at low O₂ concentration, the oxidation of deoxymyoglobin to metmyoglobin is faster than the oxidation of oxymyoglobin (Venturini, Contreras, Sarantopoulos, & Villanueva, 2006), with a higher risk in color changes during the distribution life.

To control these changes, CO at low levels has been permitted in some Countries as MAP gas for use during distribution (Cornforth & Hunt, 2008) and its application to low-oxygen packaging of fresh meat in master bags is a consolidated reality (Jeong & Claus, 2010). In this way, during storage, the bright, red color of meat is assured because CO binds strongly to myoglobin to form the extremely stable carboxymyoglobin that also reduces the oxygen consumption rate of the muscles (Seyfert, Mancini, Hunt, Tang, & Faustman, 2007).

The adoption of this master bag packaging technology without CO could be very challenging because of the management of color changes that occur during the anoxic storage (i.e. the transient

* Corresponding author. Tel.: +39 0250316659; fax: +39 0250316672.
E-mail address: sara.limbo@unimi.it (S. Limbo).

Low O₂ Master Bag for Beef Patties: Effects of Primary Package Permeability and Structure

By Eleonora Uboldi, Mirka Lamperti and Sara Limbo*

DeFENS, Department of Food, Environmental and Nutritional Sciences, Università degli Studi di Milano, via Celoria 2, 20133, Milano, Italy

The contribution of the structure and permeability of case-ready units on the colour changes of ground beef patties during low O₂ storage in master bags, aerobic blooming and display life was investigated. We selected the following case-ready solutions: PVC 8000 + closed-cell expanded polystyrene (EPS) tray, PVC 20000 + closed-cell EPS tray, linear low-density polyethylene (LLDPE) 26000 + closed-cell EPS tray and PVC 20000 + open-cell EPS tray. Patties packaged onto a closed-cell tray and wrapped with PVC 20000, but never stored in master bag, were taken as a control. We monitored oxygen depletion in the headspace of the master bags, the microbiological indexes and the appearance of the patties (colorimetric measurements and scanning images). During the storage in master bags, the use of a very high permeability wrapping film and an open-cell EPS tray allowed the best colour stability of the bottom and upper sides of patties through 7 days. After master bag opening, the same combination of materials favoured the blooming of the upper surface pigments of the product and the stability of the oxygenated pigments during the display life. Moreover, the open-cell trays helped in reducing the discolouration of the bottom side of patties. Hence, this low O₂ master bag system can enhance the colour stability of ground beef patties and therefore extend their distribution life. Copyright © 2013 John Wiley & Sons, Ltd.

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KEY WORDS: ground beef meat; low-oxygen MAP; PVC stretch film; EPS tray; blooming; shelf life

INTRODUCTION

In a recent document, the Food and Agriculture Organization of the United Nations has reported on the high levels of food waste and losses along the supply chains. Meat and meat products do not escape to this phenomenon, and the distribution and consumption represent critical stages both in industrialized and developing countries, making up approximately half of total meat losses and waste.¹ At these two levels of the food supply chain, the packaging technologies can really play a pivotal role.² In fact, not only the adoption of new materials, new processes and new storage technologies but also the optimization of consolidated materials and technologies could significantly reduce food losses, better fulfilling consumers' needs, keeping the food fresh longer and also enhancing the coordination among stakeholders in the supply chain. From this perspective, the shelf life extension strategy for meat and meat products becomes a real mandate. Different packaging solutions for the consumer sales unit are nowadays available, and the case-ready methods (i.e. products that were not repackaged in the backroom of the store) represent a new paradigm for the shelf life extension of red meat, particularly beef. In addition, labour costs and limited availability of skilled workers at retail locations will continue to drive the demand for case-ready packaging innovations.³

* Correspondence to: Sara Limbo, DeFENS, Department of Food, Environmental and Nutritional Sciences, Università degli Studi di Milano, via Celoria 2, 20133 Milano Italy.
E-mail: sara.limbo@unimi.it