POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES

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Pol. J. Food Nutr. Sci.
2010, Vol. 60, No. 2, pp. 115-120

ASSESSMENT OF THE SHELF-LIFE OF MEAT PACKED IN VARIOUS PROTECTIVE ATMOSPHERES WITH ADDITION OF LYSOZYME

Bożena Danyluk[1], Beata Mikołajczak[1], Bożena Grześ[1], Edward Pospiech[1,3], Ryszard Kowalski[1], Piotr Konieczny[2]

[1]Institute of Meat Technology, [2]Department of Food Quality Management; Poznan University of Life Sciences, Poznań, Poland; [3]Meat and Fat Research Institute in Poznan, Poznań, Poland

Key words: meat, modified atmosphere, microbiology, hydrophobicity, lysozyme

The objective of this research was to evaluate the impact of different protective atmospheres, with or without the addition of lysozyme, which were used to pack swine *longissimus* muscles kept in cold store conditions for the period of up to 3 weeks. The employed atmospheres comprised: vacuum, modified atmosphere with carbon oxide (70% CO_2, 29.5% N_2, 0.5% CO) MAP-CO and with oxygen (79% N_2, 20% CO_2, 1% O_2) MAP–O_2. The evaluation of the shelf life of packed meat was performed on the basis of microbiological examination determining the total number of aerobic bacteria, *Enterobacteriaceae* family bacteria, enterococci as well as lactic acid bacteria (LAB). Measurements of hydrophobicity of muscle centrifugal drip protein were employed as a method allowing to compare the microbiological status of the meat during its storage in the chilled room. The atmospheres applied were found not to differ with regard to the degree of aerobic microorganisms development in the stored meat. The application of vacuum or MAP–O_2 was found as a best way to inhibit the development of LAB. Samples with the addition of lysozyme were characterised by a slower development of microorganisms than these without its addition only in the case of samples packed in MAP–O_2 and stored for 21 days. Storage time exerted a significant influence on the hydrophobicity of meat, which was lower in vacuum conditions than when protective gases were applied. The inclusion of lysozyme resulted in an increase of hydrophobicity.

INTRODUCTION

Stable and safe storage of fresh meat is impossible nowadays without the application of appropriate packaging as well as additional protective agents. One of the problems is the selection of correct packaging and a protective agent that would allow the longest storage of meat and, simultaneously, assure safety to consumers. Majority of microorganisms causing food putrefaction are aerobes, which means that their development may be inhibited by restricting the access of oxygen. Hence the idea of meat preservation by packing it into foils and, simultaneously, ensuring vacuum (vacuum packaging) or appropriate gas environment of specific chemical composition inside (modified atmosphere packaging – MAP). The advantages of this method consist not only in inhibiting the development of aerobic microflora but, equally importantly, in restricting oxidation processes leading to undesirable changes in taste and colour[McMillin, 2008]. Meat packed in vacuum or in modified atmosphere must be stored in chilled room conditions for a low redox potential of meat and environmental conditions enable the growth of anaerobic bacteria. Failure to observe these principles leads to putrefaction of meat caused by the growth of *Clostridium botulinum* and creates conditions for the development of type E botuline toxin [Beets & Gaze, 1995].

Several years of practical application of modified atmosphere have changed the view as to the use of gas mixtures with a high oxygen content to pack fresh meat. Originally, modified atmosphere with a high level of oxygen was readily employed due to a durable improvement in meat colour during storage. However, investigations carried out in recent years [Huff-Lonergan & Lonergan, 2005; Sørheim, 2006] revealed a negative impact of packaging in the atmosphere rich in oxygen. Meat was observed to go rancid, thus the process was found to require the use of antioxidants which are not accepted in many countries. Oxidation exerts a negative influence on meat water holding capacity and slows down the process of its tenderisation [Huff-Lonergan & Lonergan, 2005]. An additional argument against the use of such an atmosphere is a high risk posed by the application of gas mixtures with such a high oxygen content due to their flammability.

The interest in carbon oxide (CO) as a component of MAP employed in the packing of meat as well as its products has been steadily growing in recent years [Sørheim, 2006]. Several years ago, tuna fish vacuum packed and deep-frozen meat which was additionally treated with CO was marketed in some EU countries. The most apparent feature of this product was its cherry-red colour. A positive impact of carbon monoxide on muscle tissue colour has been known for over one hundred years and is protected by a patent. This method takes advantage of the fact that CO binds with the hem from myoglobin or hemoglobin and forms a light-red protein complex. This leads to the stabilisation of meat colour, especially on its surface [Stenzel & Feldhusen, 2004].

Author's address for correspondence: Bożena Danyluk, Institute of Meat Technology, Poznan University of Life Sciences, Wojska Polskiego 31, 60-624 Poznań, Poland; tel.: (48 61) 848 72 60; e-mail: danyluk@up.poznan.pl

Lysozyme ensures an additional preservation of products which were vacuum-packed or packed in modified atmosphere. The basic property of lysozyme is its capability to dissolve cell membranes of Gram-positive bacteria. There is a large group of pathogenic bacteria sensitive to lysozyme including: *Salmonella, Brucella, Klebsiella, Shigella, Neisseria, Pseudomonas, Pasteurella, Erwinia, Escherichia,* as well as *Bacillus, Staphylococcus (aureus), Clostridium (botulinum)* and *Listeria (monocytogenes)*. Lysozyme inhibits significantly the development of proteolytic strains of *Clostridium botulinum*, whereas non-proteolytic B, E and F types are relatively resistant to lysine. The spectrum of the antibacterial action of lysozyme can be broadened considerably when antibiotics are applied simultaneously, which is the result of the synergistic effect. However, their addition as food additives is strictly prohibited. Moreover, lysozyme is known to possess a wide spectrum of antiviral activities, including activity against a group of carcinogenic viruses [Trziszka & Kopeć, 1997a].

In addition, lysozyme turned out to be a good bio-preservative in such meat products as: salami, cooked sausages, ground raw meat, semi-dried products. It was also demonstrated that treatment with lysozyme, nitrites and salt was particularly favourable [Kijowski & Leśnierowski, 2000]. Having in mind economic profitability as well as the product's quality and shelf-life, employment of the immersion technique, *i.e.* short dipping of product in lysozyme solution, has been recommended by some authors [Trziszka & Kopeć, 1997a].

Development of microorganisms on meat is not only connected with the type of the protective atmosphere. Researches of Benito *et al.* [1997] show a significant correlation between the cell surface hydrophobicity and the strength of attachment of the bacteria to the muscle surface. Therefore, it may be interesting to confirm the relationship between the hydrophobicity of muscle proteins and the number of microorganisms causing the spoilage of meat.

The aim of the study was to assess the microbiological stability of meat packed in different protective atmospheres (vacuum, MAP-CO, MAP-O_2) with or without the addition of lysozyme and stored in cold room for the period of three weeks. Additional determinations were conducted to examine the fluorescence of protein of centrifugal drip from this meat and to investigate changes in protein hydrophobicity using the fluorescence method as affected by the microbiological status of the samples examined.

MATERIALS AND METHODS

Meat

The experimental material comprised the *longissimus* muscle (LM) cut out from swine carcasses. Meat for analyses was selected in such a way as to eliminate PSE defect and acid meat. The selection was performed on the basis of measurements of the pH value 45 min and 24 h after slaughter as well as electrical conductivity 90 min and 24 h after slaughter employing the classification proposed by Borzuta & Pospiech [1999], (Table 1). Measurements of pH were carried out using a HANDYLAB 2 pH-meter of the Schoot Company equipped in a combined electrode type Blue Line 21, whereas electrical conductivity was measured using an LF/PT-STAR device.

Packing procedure

Each time, six pieces of the *LM* were packed. Each muscle was divided into 4 parts (25 cm each) which allowed performing analyses after four periods of storage. Finally, 6 variants were obtained which comprised: vacuum packing with or without lysozyme (vacuum and vacuum + lysozyme); packing in the atmosphere with oxygen with or without lysozyme (MAP-O_2 and MAP-O_2 + lysozyme) and packing in the atmosphere with carbon monoxide with or without lysozyme (MAP-CO and MAP-CO + lysozyme). The experiment was repeated three times. The composition of gas mixtures which were used as protective atmospheres was as follows: MAP-O_2 mixture: nitrogen – 79%, carbon dioxide – 20% and oxygen – 1%; and MAP-CO mixture: carbon dioxide– 70%, nitrogen – 29.5% and carbon monoxide – 0.5%.

Half the samples were covered with lysozyme which was distributed uniformly on the surface of the entire sample by spraying 2.3 g per each meat sample. An enzymatic preparation Delvozyme–L was used in the described experiment. Its active substance was lysozyme of chicken egg – E 1105. The activity of lysozyme accounted for 9000 units/mL.

After this treatment, the samples were placed in bags using a TEPRO PP 15 type packing machine. In the case of the vacuum-packed samples (with or without lysozyme addition), the machine was programmed so as to ensure 98% vacuum inside the pack.

Bags used for packing (21 × 29 cm in size) were made of polyamide and polyethylene and were characterised by the following permeabilities: oxygen 16–19 $cm^3/m^2 \times 24$ h, carbon dioxide 100–130 $cm^3/m^2 \times 24$ h, nitrogen 3–5 $cm^3/m^2 \times 24$ h, and water vapour 2–3 $g/m^2 \times 24$ h.

Microbiological assessment

The packed meat was stored at chilled room conditions (4°C) and changes in its microbiological status were determined after consecutive storage periods, *i.e.* after 2, 7, 14 and 21 days. The performed microbiological evaluation included determinations of: total number of mesophilic aerobic bacteria (MAB), lactic acid bacteria (LAB), enterococci and *Enterobacteriaceae* family bacteria. Individual cultures were obtained using the plate count on the following media by BTL Company:

- nutrient agar – determination of the total number of microorganisms (incubation for 48 h, at 30°C),
- VRBL – determination of the number of *Enterobacteriaceae* family bacteria, including *Coli* group bacteria (incubation for 24 h, at 30°C),
- Slanetz and Bartley – determination of the number of enterococci (incubation for 48 h, at 37°C),
- MRS – determination of the number of LAB (incubation for 72 h, at 30°C).

Readings and calculations of the results obtained were performed according to the Polish Standard [PN-A-82055-6:1994]. Experiments were repeated three times.

Determination of protein surface hydrophobicity

Levels of meat hydrophobicity S_o were determined with the assistance of the method based on measurements of fluorescence using an ANS reagent (8-anilino 1-naphthalene sul-

fonic acid) of the Sigma Aldrich Company [Li–Chan *et al.,* 1984]. After 48 hours, 1, 2 and 3 weeks of storage, muscle juice was collected for analyses from the foil bags in which meat was stored. Once the protein content was assessed, the juice was appropriately diluted (0–1 mg/mL) and its hydrophobicity was determined. Relative intensity of fluorescence at the wave lengths of 390 nm (excitation) and 490 nm (emission) was measured in samples with known protein concentrations using a Luminescence Spectrometer, type LS 55 (Perkin Elmer). Surface hydrophobicity S_o constituted the directional coefficient of the determined straight line.

Statistical analysis

Results obtained in the study were subjected to a statistical analysis. Research hypotheses were verified at the level of significance $p \leq 0.05$ with ANOVA two-factorial analysis of variance and Tukey's test of multiple comparisons. All calculations were carried out using STATISTICA 8.0 computer software.

RESULSTS AND DISCUSSION

Data presented in Table 1 indicate that all samples were of normal quality.

Table 2 presents results of the microbiological analyses performed. The number of aerobic bacteria in the first period of assessment (2 days) was similar in all the examined samples and the log of this number was 2.27-2.28. Numbers of these bacteria continued to grow in the consecutive periods of cold storage but the statistical analysis performed failed to show any differences. The determined log ranged from 3.61 to 4.24 after 21 days of cold storage (Table 2).

Current requirements found in the EU Directive from the 15th of November 2005 do not specify the allowable number of aerobic bacteria in 1 g of meat cut out from the carcass [Commission Regulation (EC) No 2073/2005]. The above Directive stipulates only the number of bacteria on the surface of swine carcass. It should not exceed the maximum value of 4.0-5.0 log cfu/cm^2. Therefore, the results obtained in this experiment can be considered satisfactory because it is commonly believed [Kołożyn-Krajewska, 2003] that aerobic bacteria count of 10^6 per 1 g of meat does not pose health risk. Also other studies [Zaleski, 1985] indicate that if the total bacterial count in raw meat after slaughter and at initial processing does not exceed 10^4-10^5/g, then such meat is acceptable.

The results obtained in the presented study failed to show that the method of packing changed significantly the level of meat contamination with aerobic bacteria. Nevertheless, some researchers [Skandamis & Nychas, 2002] claim that the effectiveness of packing is greater in modified atmosphere than in vacuum. On the other hand, results published by García-Esteban *et al.* [2004] revealed that in the case of hams pickled using the "dry" method and stored for 8 weeks, the microbiological status of the product did not depend on the method of packing (vacuum and MAP).

Carbon monoxide applied in the modified atmosphere did not influence the growth of aerobic bacteria in the samples (MAP-CO), similarly to the samples which did not contain CO (MAP-O_2). Investigations on the effect of carbon monooxide on bacteria development were also carried out by Luño *et al.* [1998]. Their results proved that CO inhibited the growth of mesophilic aerobic bacteria when applied at a greater concentration (1%) than in the case of this study and only when, simultaneously, the content of CO_2 in the atmosphere reached 50%. This effect was not recorded when, at 1% proportion of CO in the atmosphere of protective gases, carbon dioxide constituted only 20%.

The number of LAB was increasing during cold storage (Table 2). Statistically significant differences were demonstrated after 21 days of storage. The greatest number of bacteria was recorded in the samples packed in MAP-CO, MAP-CO + lysozyme and MAP-O_2. Log of the number of these bacteria ranged 3.16 to 3.26, whereas the statistical analysis performed showed that the contamination with LAB determined in the above mentioned variants was significantly greater than in the samples packed in vacuum and MAP-O_2 + lysozyme. The investigations performed demonstrated that the application of vacuum without lysozyme as well as MAP-O_2 + lysozyme failed to cause a significant increase in the number of acidifying bacteria during a three-week long storage of the *longissimus dorsi* muscle in cold store conditions. It suggests that by a small initial microbial contamination of meat, the antimicrobial activity of lysozyme becomes evident only when the population of LAB increases. In our case, it was observed after three weeks of cold storage when the log of the number of these bacteria in control packed meat reached the level above 3. Lysozyme was found to inhibit the development of LAB only in the case of meat packed in MAP-O_2 and stored for three weeks. Despite numerous reports indicating the bacteriostatic action of lysozyme

TABLE 1. Electrical conductivity (EC) and pH value in the examined muscles.

Parameters	Time	Sample type					
		Vacuum		MAP-CO*		MAP-O_2	
		vacuum	vacuum + lysozyme	MAP – CO	MAP – CO + lysozyme	MAP – O_2	MAP – O_2 + lysozyme
pH	45 min	$6.23^{a*} \pm 0.19$	$6.15^{abcd} \pm 0.12$	$6.23^{ad} \pm 0.30$	$6.29^{a} \pm 0.30$	$6.17^{abcd} \pm 0.16$	$6.18^{acd} \pm 0.19$
	24 h	$5.65^{bc} \pm 0.14$	$5.78^{abcd} \pm 0.23$	$5.78^{abcd} \pm 0.21$	$5.74^{abcd} \pm 0.08$	$5.67^{bcd} \pm 0.10$	$5.61^{b} \pm 0.01$
EC (mS/cm)	90 min	$2.65^{ab} \pm 0.61$	$2.65^{ab} \pm 0.61$	$3.23^{ab} \pm 0.57$	$3.23^{ab} \pm 0.57$	$2.82^{ab} \pm 0.43$	$2.82^{ab} \pm 0.43$
	24 h	$1.67^{a**} \pm 0.03$	$1.67^{a} \pm 0.03$	$3.9^{b} \pm 1.56$	$3.9^{b} \pm 1.56$	$2.7^{ab} \pm 0.35$	$2.7^{ab} \pm 0.35$

*$p \leq 0.05$; ** – explanation of abbreviations are given in the "Material and Methods" section.

TABLE 2. Results of microbiological assessment of meat.

Time (days)	Sample	Bacteria count in 1 g (log) ($\bar{x}$ ± SD)			
		aerobic bacteria	LAB	enterococci	*Enterobacteriaceae* familia bacteria
2	Vacuum	2.27±1.11^{abcd}	1±0^{a*}	ni/sc	ni/sc
	Vacuum + lysozyme	2.28±1.11^{abcd}	1±0^{a}	ni/sc	ni/sc
	MAP-CO	2.28±1.11^{abcd}	1±0^{a}	ni/sc	ni/sc
	MAP-CO+ lysozyme	2.28±1.11^{abcd}	1±0^{a}	ni/sc	ni/sc
	MAP-O_2	2.28±1.11^{abcd}	1±0^{a}	ni/sc	ni/sc
	MAP-O_2+ lysozyme	2.28±1.11^{abcd}	1±0^{a}	ni/sc	ni/sc
7	Vacuum	1.96±0.39^{ab}	1±0^{a}	ni/sc	ni/sc
	Vacuum + lysozyme	1.66±0.58^{a}	1±0^{a}	ni/sc	ni/sc
	MAP-CO	2.17±0.19^{abc}	1±0^{a}	ni/sc	ni/sc
	MAP-CO+ lysozyme	2.16±0.21^{abc}	1±0^{a}	ni/sc	ni/sc
	MAP-O_2	2.56±0.54^{abcd}	1±0^{a}	ni/sc	ni/sc
	MAP-O_2+ lysozyme	2.48±0.24^{abcd}	1.16±0.28^{ab}	ni/sc	ni/sc
14	Vacuum	3.16±0.15^{abcd}	2.10±0.95^{abc}	ni/sc	ni/sc
	Vacuum + lysozyme	3.19±0.17^{abcd}	2.04±0.91^{abc}	ni/sc	ni/sc
	MAP-CO	3.29±0.11^{abcd}	2.42±0.81^{abc}	ni/sc	ni/sc
	MAP-CO+ lysozyme	3.27±0.09^{abcd}	2.05±0.91^{abc}	ni/sc	ni/sc
	MAP-O_2	3.51±0.51^{abcd}	2.21±0.99^{abc}	ni/sc	ni/sc
	MAP-O_2+ lysozyme	3.28±0.21^{abcd}	1.99±0.99^{abc}	ni/sc	ni/sc
21	Vacuum	3.80±0.40^{bcd}	3.03±0.46^{ab}	ni/sc	ni/sc
	Vacuum + lysozyme	3.61±0.58^{abcd}	2.85±0.62^{abc}	ni/sc	ni/sc
	MAP-CO	4.04±0.57^{cd}	3.26±1.03^{c}	ni/sc	ni/sc
	MAP-CO+ lysozyme	4.09±0.48^{cd}	3.22±0.90^{c}	ni/sc	ni/sc
	MAP-O_2	4.24±0.25^{d}	3.16±0.97^{c}	ni/sc	ni/sc
	MAP-O_2+ lysozyme	3.98±0.49^{cd}	3.03±0.99^{ab}	ni/sc	ni/sc

ni/sc – no increase or single colonies; *p≤0.05.

[Kijowski & Leśnierowski, 1995; Trziszka & Kopeć, 1997b; Danyluk & Kijowski, 2001], the spraying of experimental samples with the above-mentioned enzyme failed to inhibit the development of aerobic or lactic acid bacteria. It was likely to be due to a relatively low microbiological contamination of the samples examined as no enterococci and *Enterobacteriaceae* family bacteria were detected in any of the meat samples assessed. The microbiological contamination with LAB was observed to increase during storage so that after 3 weeks it reached the level of $\log_{10}$ 2-3. The development of these bacteria in meat during storage in vacuum and modified atmosphere was also reported in other papers [Nissen *et al.,* 1996; Labadie, 1999] because at a reduced redox potential, LAB become predominant microflora. It is evident from investigations carried out by Nissen *et al*. [1996] that during the storage of beef under conditions of vacuum and MAP, the numbers of LAB varied depending on the method of packing (vacuum, MAP) and storage temperature (2-6°C) and amounted to $\log_{10}$ 5-7 after 5 weeks of storage.

The results obtained show that a good microbiological quality of meat stored for a long time can be maintained by keeping high hygienic standards and by reducing the initial microbiological contamination of its surface.

The available literature data [Kim *et al.,* 2003] indicate that it is possible to utilise fluorescent measurements to monitor the level of contamination with *Coli* group bacteria. However, in the investigations performed, no unequivocal correlations were found between the applied variants of packing, storage time and the presence of aerobic and lactic acid bacteria.

Values of surface hydrophobicity presented in Figure 1 illustrate the course of fluorescence variability in muscle juice proteins during storage and in relation to the applied method of packing with or without lysozyme. Because residues of aromatic amino acids (phenylalanines, tyrosines, tryptophan) react with ANS, this hydrophobicity is frequently referred to in literature as aromatic hydrophobicity [Nakai *et al.,* 1996; Wilkins, 1985].

It was established that proteins from the muscle juice obtained from the meat packed in modified atmosphere MAP-O_2 supplemented with lysozyme (Figure 1) were characterised by the highest surface hydrophobicity (368.51-583.64). On the other hand, the vacuum-packed samples were charac-

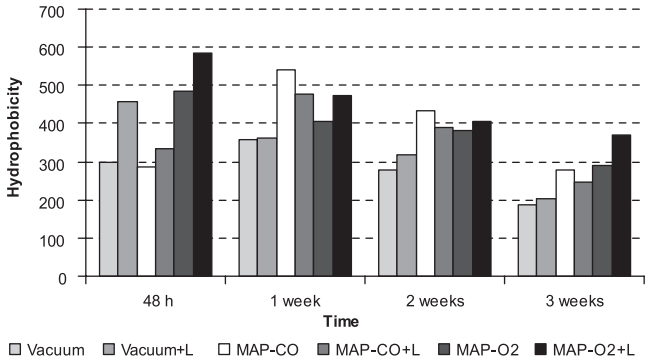

FIGURE 1. Hydrophobicity changes of centrifugal drip in relation to the type of protective atmosphere and time of storage.

terised by the lowest hydrophobicity, the value of which decreased from 299.69 after 48 h to 187.79 in the last period of analyses (Figure 1).

The performed analysis of variance showed that storage time exerted a significant influence on aromatic hydrophobicity irrespective of the packing variant applied. Mean hydrophobicity determined in the last analytical period amounted to 256.38 and was significantly lower in comparison to the values determined after 48 h and 1 week.

A significantly lower (278.89) hydrophobicity of the samples derived from the vacuum-packed meat was observed in comparison with those packed using the MAP-O_2 atmosphere and lysozyme (458.0). Packing in the atmosphere containing 70% CO_2, 29.5% N_2 and 0.5% CO with or without the addition of lysozyme had no significant effect on sample hydrophobicity despite the fact that the application of CO with lysozyme usually somewhat limited the growth of lactic acid bacteria.

The above appears to indicate that the development of microflora caused a reduction in hydrophobicity. However, this process depended on the composition of the protective atmosphere. Under vacuum conditions, hydrophobicity was lower than when a mixture of protective gases was used. As a rule, the addition of lysozyme evoked an increase in this indicator.

A significant correlation was demonstrated between hydrophobicity and the log of aerobic acid bacteria ($r=-0.54$; $p\leq0.05$) and LAB ($r=-0.61$; $p\leq0.05$).

CONCLUSIONS

1. The application of vacuum or MAP–O_2 was found the most effective in inhibiting the development of LAB.

2. Samples with the addition of lysozyme were characterised by a slower development of microorganisms than the samples without its addition, but this difference was statistically significant only in the case of samples packed in MAP–O_2 and stored for 21 days.

3. When the contamination with bacteria was very low, no differences were observed in the inhibition of the growth of aerobic microorganisms in the stored meat packed in various protective atmospheres.

4. Storage time exerted a significant influence on the reduction of hydrophobicity of drip protein from packed meat irrespective of the applied protective atmosphere and statistically significant differences occurred between the samples stored for 48 hours and 7 days and those analysed after three weeks.

5. The growth of microflora leads to the reduction of sample hydrophobicity, however, this process was found to depend on the composition of the protective atmosphere.

6. Hydrophobicity was lower in vacuum conditions than when mixtures of protective gases were applied, and the inclusion of lysozyme resulted in its increase.

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Received April 2009. Revision received October and accepted November 2009.