EFFECT OF DIFFERENT TYPES OF PACKAGING ON THE QUALITY OF MINCED PORK MEAT WITH BIOACTIVE COMPONENTS AND LACTIC ACID

Anita Rokaitytė[1], Gintarė Zaborskienė[1,2], Sonata Orentaitė–Gustienė[1], Justina Šiupinienė[1]

[1]Lithuanian University of Health Sciences, Tilžės st. 18, LT– 47181 Kaunas, Lithuania
[2]Food Institute, Kaunas University of Technology, Radvilėnų st. 19, LT–51180 Kaunas, Lithuania

**Corresponding author: Anita Rokaitytė*
E–mail: anita.rokaityte@lsmuni.lt; tel. +370 62 328294

Abstract. The hurdle technology, which consists of combining different preservative techniques is an alternative way of preventing growth of spoilage microorganisms while keeping a high quality of flavor. The objective of the study was to enhance the quality of fresh minced pork meat with mixture of lactic acid (LA), natural antioxidant dihydroquercetin (DHQ) from Siberian larch (*Larix sibirica Ledeb*) and essential oil (EO) extract of thymol (TH), using combinations of different packaging (vacuum (VP), modified atmosphere (MAP) and aerobic packaging (AP)). In the course of the study the aerobic colony count (ACC), *Escherichia coli,* yeast and mold counts and formation of biogenic amines (BA) during 9 days of storage at +4 °C temperature were observed. VP and MAP effectively inhibited ACC, yeast and mold counts and level of BA in minced pork meat ($P<0.05$) during 9 days of storage at +4 °C temperature. LA and its mixtures with DHQ and TH statistically significantly reduced the ACC, *E. coli* count, total amount of BA and meat pH (in all cases $P<0.05$) in comparison with control samples in VP. In general, results suggest that adding a combination of all three additives resulted in a product with a distinctly longer shelf life in VP. These results could arise as an interesting approach for the improvement of food preservation using more natural procedures, considering the current demand of consumer and sensory quality of foods.

Keywords: pork, modified atmosphere packaging, dihydroquercetin, thymol, lactic acid, microorganisms

Introduction. Synthetic preservatives are widely used by the meat industry to control the growth of spoilage and pathogenic microorganisms to extending the shelf-life, quality and safety of meat products. However, the use of natural antimicrobial compounds in food has gained much attention by the consumers and the food industry. This is due primarily to two major factors. First, the misuse and mishandling of antibiotics has resulted in the dramatic rise of a group of microorganisms including foodborne pathogens that are not only antiobiotic resistant but also more tolerant to several food processing and preservation methods (Gyawali and Ibrahim, 2014; Artem'eva et al., 2015). In addition, increasing consumers awareness of the potential negative impact of synthetic preservatives on health versus the benefits of natural additives has generated interest among researchers in the development and use of natural meat products. This has prompted the meat industry to look for alternative preservatives that can enhance the safety and quality of products. Compounds derived from natural sources have the potential to be used for meat products safety due to their antimicrobial properties (Anand and Sati, 2013).

Natural antimicrobials, including plant extracts and their EOs have been shown to have the potential for use as alternatives to chemical antimicrobials. The antimicrobial activities of some EOs are mainly due to the presence of some major bioactive compounds, including phenolic acids, terpenes, aldehydes and flavonoids (Dinesh, 2013; Aziz and Karboune, 2016).

The DHQ (taxifolin) is a member of the group of flavonons (Vladimirov et al., 2009). The satisfactorily pure DHQ may be extracted from Siberian larch (*Larix sibirica Ledeb*) and has a positive effect on human health (Trouillas et al., 2004; Tarahovsky et al., 2007). DHQ is used for application in food products as dietary ingredients, natural antioxidants, food additives and preservatives, to enhance the keeping quality or stability of a food, without changing the nature, substance or quality of the food (Ivanov et al., 2009; Wang et al., 2011).

LA is a commonly occurring organic acid, which is valuable due to its wide use in food and food-related industries (Scott et al., 2015). LA is used mainly as an acidulant but is also important in the ready-to-eat meat industry and as food fortifiers and pH adjusters. LA is widely used in fresh and processed meats; extending the shelf life and inhibiting the growth of *E. coli* and *Listeria*, *Salmonella* among others (Wee et al., 2006). LA is shown antimicrobial activities against organisms because of its abilities to reduce pH level, exert feedback inhibition and interfere with proton transfer across cell membranes (Davison et al., 2005).

One of the traditional ways of controlling microbial growth in these products, thus, improving safety and delaying spoilage is application of EOs (Dinesh, 2013). EOs can be combined with other antimicrobial compounds and/or other preservative technologies to obtain a synergistic effect without compromising antimicrobial activities (Nguefack et al., 2012). Other authors suggested that the combined use of MAP and EOs may be commercially applicable for improving the preservation of meat-based products and extending shelf life by 5–6 days (Chouliara et al., 2007; Mastromatteo et al., 2009).

Several studies related to natural antimicrobials have demonstrated the efficacy of plant-derived compounds in

food applications, as well as factors influencing this effectiveness (Gyawali and Ibrahim, 2012; Hayek et al., 2013). However, there has been limited research related to the structural-functional relationship of these compounds. As a result, the importance of the chemical composition of plant-derived compounds with regard to their antimicrobial activity is still not well understood.

The objective of the study was to enhance the quality of fresh minced pork meat using combination of different packaging (AP, VP and MAP) with LA and its mixtures with DHQ and TH on microorganisms found in minced meat and formation of BA.

Materials and methods

Meat samples

The experimental material consisted of pork meat (shoulder) collected directly from a slaughterhouse one day after slaughtering. The experimental material were transported to the laboratory at 4 °C and minced with a sterilized meat mincer in 3 mm size. Minced meat samples were divided into 12 samples (12x0.5 kg) considering different treatments with LA and bioactive substances. The samples were named as follows: (I) DHQ+LA+TH, (II) DHQ+LA, (III) LA and (IV) not treated control group.

Preparation of bioactive components solutions for the antibacterial properties analyses

Powder concentrate of DHQ (99.4%), extracted from Siberian larch *(Larix sibirica Ledeb)* and produced by the company Flavit Ltd, Pushtino (Russia) was used. DHQ was diluted using 35 °C distilled water to make 10 ml of 0.024% (w/v) DHQ aqueous solution.

TH (99.5%) and LA (50.0%) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and kept at 4 °C. All of the solutions (each of them 10 ml) were made on the day of the research: 0.003% (w/v) TH aqueous solution and 0.5% (w/v) LA aqueous solution.

Packaging parameters

The minced meat samples were weighed and packed using the three different packaging methods: VP, MAP and AP. VP and MAP were performed using a Multivac R230 model 542 packaging machine (Multivac, Wolfertschwenden, Germany). The vacuum bag (CTHgvac 90, Curevac AB, Göteborg, Sweden) was 90 µm thick with transmission rates (cm^3/m^2, 24 h, 23 °C): O_2 40, CO_2 150; MAP was filled with gas composed of 80% O_2 and 20% CO_2. Control samples (AP) were packaged under atmospheric air without using any gas composition. The samples were stored in the dark under refrigerated conditions (+ 4 °C) for 9 days. Analyses of pH, microorganisms and BA were carried out on the 1st, 3rd, 5th, 7th and 9th day of storage. The whole experiment was replicated three times.

Microbiological analysis

Samples of 10 g were taken at random for each sample and aseptically weighed into a sterile stomacher bag with 90 ml of sterile Buffered peptone water 0.1% (w/v) (REF 611014, Liofilchem, Italy) and homogenized for 1 minute in a model 400 Stomacher (Seward Medical, London, UK). Serial decimal dilutions were made and ACC count were enumerated by plating on Plate Count Agar (REF 610040, Liofilchem, Italy) at 30 ºC for 72 hours; *E. coli* were enumerated by plating on Tryptone Bile X-Glucuronide Medium Agar (REF 4021562, Biolife, Italy) at 37 ºC for 24 hours; yeast and mold were enumerated by plating on Sabouraud Caf Agar (REF 610203, Liofilchem, Italy) at 30 ºC for 48 hours.

Microbiological data were transformed into logarithms of the number of colony forming units (cfu/g).

Detection of biogenic amines

A reversed-phase high-performance liquid chromatography (RP-HPLC) method was used for the quantitative analysis of the BA – tryptamine, phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermidine, and spermine. BA were extracted from a homogenized sample with 0.4 mol/l perchloric acid. The derivatization of samples was carried out using the modificated methodology of Ben-Gigirey et al. (2000). Extract was derivatised for 45 min by dansyl chloride (5-dimethylaminonaphtalene-1-sulfonylchloride) solution in acetone at 40 ºC. The samples were filtered through 0.45 µm membrane filter (Millipore Co., Bedford, MA, JAV), 10 µl was injected into chromatographic system (Aligent 1200 Series, Germany). Analysis was performed using LiChro column CART® 95 125-4. Carrier phase – eluents: B – acetonitrile, A – ammonium acetate 0.1mol/l. Analysis lasted 28 min changing the content of eluents during the first 19 min from 50 % of B to 90 % of B (from 50 % of A to 10 % of A respectively), then leaving the content constant for 1 min – 90 % of B. Later, to ensure isolation of materials for another analysis, eluent with the composition of 50 % of B and 50 % of A was being added to the chamber for 8 minutes. The flow rate of 0.9 ml/min did not change during analysis, column temperature 40 ºC. UV detection was observed at 254 nm. BA were identified by comparing the retention time of each amine in the chamber with the retention time of the respective reference material. Internal standard method of calculating the peak area for the defined amount of reference material was used to perform quantitative analysis. The limit of detection is between 0.02–0.1 µg/ml for different BA.

pH measurement

pH was measured on the surface of all samples according to the standard method for determination of meat pH: LST ISO 2917:2002. The average pH of the sample was determined. pH measurements were carried out using PP-15 pH-meter (Sartorius Professional meter for pH Measurement, Germany).

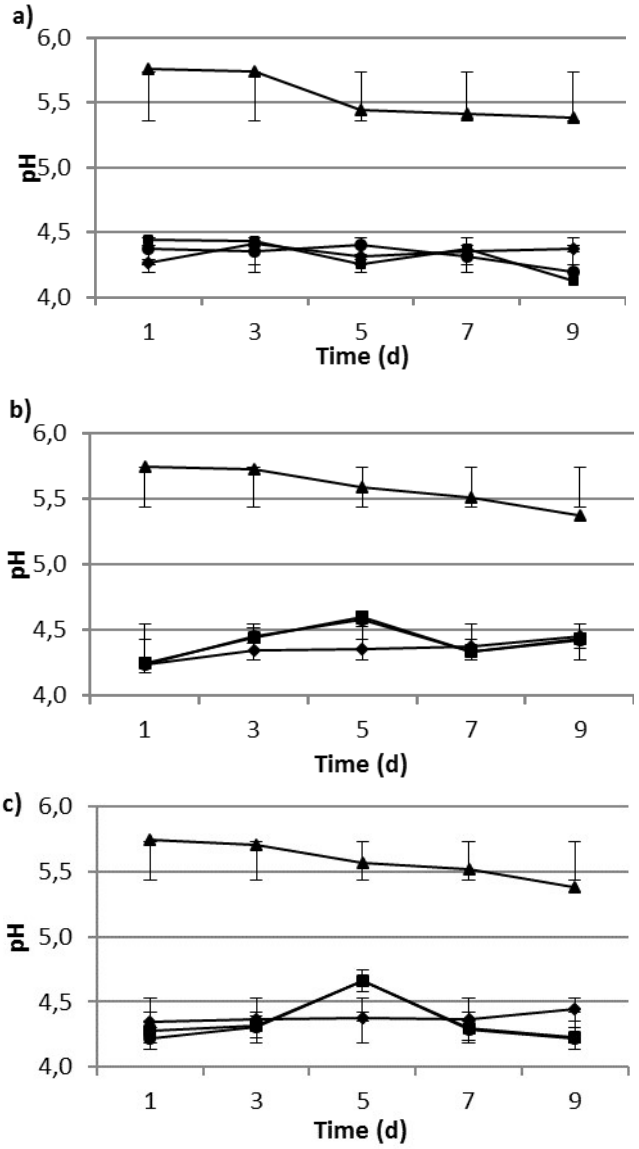
Statistical analysis of the data

Data were statistically analyzed using the SPSS 20.0 software (SPSS Inc., Chicago, Illinois, USA). Differences between dates were evaluated by the analysis of variance method (one-way ANOVA) with a significant level of $P<0.05$ (Draper and Smith, 1998). Multiple comparisons were estimated by Fishers Least Significant Difference method and Dunnet test was applied when control group was present. Student's t test was used to determine average values of indicators, standard deviations (SD) and

linear correlations. The correlation was considered reliable when P<0.05.

Results

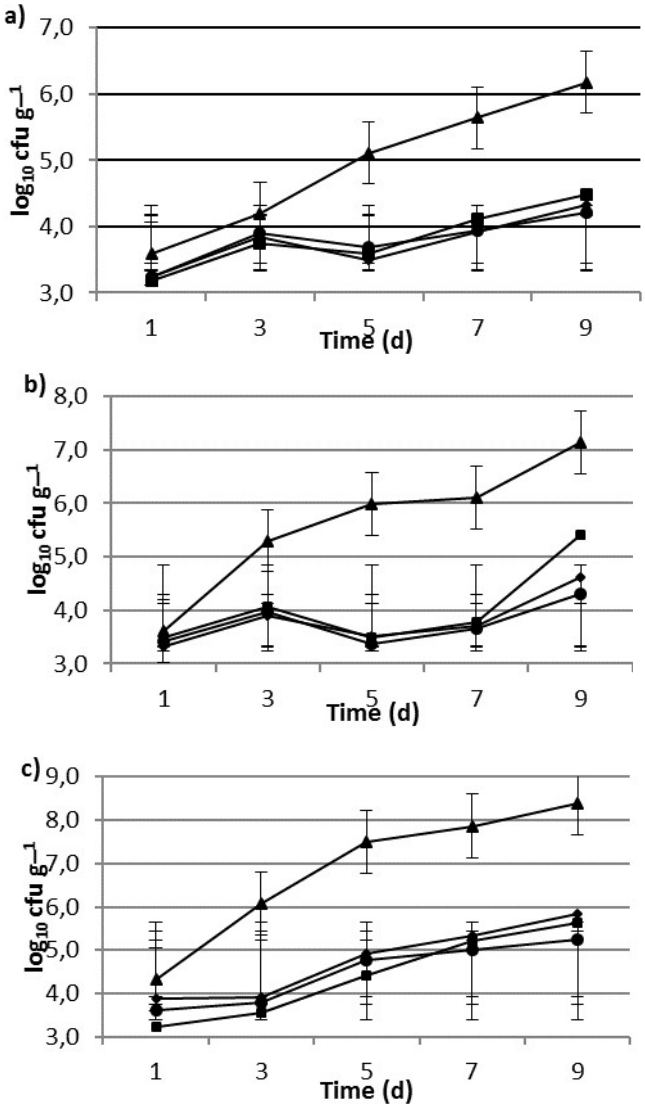
Significant differences were observed between the pH values of control meat and treated samples throughout storage. However, there were no significant differences (P>0.05) in pH among the different treatments and packaging (Fig. 1).



(a) VP, (b) MAP, (c) AP without (▲), and with (♦) DHQ+LA+TH, (■) DHQ+LA and (●) LA

Figure 1. **Variation of the pH mean values of minced pork meat during 9 days of storage at +4 ^{0}C**

Results demonstrated that there were significant differences (P<0.05) in the ACC count when different packaging were used (Fig. 2). ACC count, in control sample, reached 5.64 log_{10}cfu/g^{-1}, 6.11 log_{10}cfu/g^{-1} and 7.86 log_{10}cfu/g^{-1} for VP, MAP and AP packaging respectively after 1 week of storage. LA and its mixtures with DHQ and TH effectively inhibited bacterial growth or reduced numbers of viable bacteria (P<0.05) compared to control sample. In general, the antimicrobial activity of these mixtures increased when the pH decreased.



(a) VP, (b) MAP, (c) AP without (▲), and with (♦) DHQ+LA+TH, (■) DHQ+LA and (●) LA

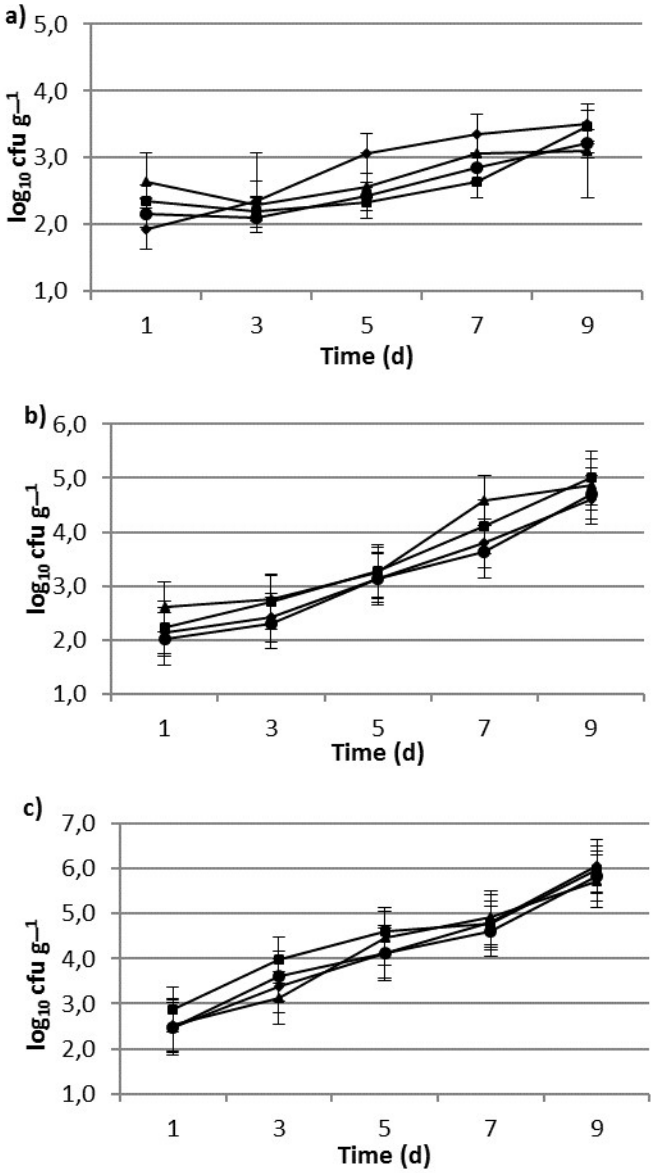
Figure 2. **Variation of the aerobic colony count in minced pork meat during 9 days of storage at +4 ^{0}C**

Results demonstrated that VP and MAP effectively inhibited yeast and mold in minced pork meat during 9 days of storage (P<0.05). However, there were no significant differences (P>0.05) in the total yeast and mold count among the different treatments.

LA and its mixture with TH and DHQ, was distinguished by a strong synergistic effect and statistically significantly reduced the *E. coli* count after 3 days in all types of packaging (Fig. 4). Nevertheless, there were no significant differences (P>0.05) in the *E. coli* count among the different packaging.

The packaging conditions (VP and MAP) had a significant effect of BAs during 9 days period (P<0.05) (Fig. 5). The significant difference of the total BAs content was determined between DHQ+LA and control

samples (P<0.05) in all different packaging. The total amount of BAs was significantly lower in VP, than in the sample with DHQ+LA packed in MAP (P<0.05) and in AP (P<0.05). Concerning the concentration of single BAs during the shelf life study, no relevant differences between different treatment and packaging were reported for tryptamine, phenylethylamine, tyramine, spermidine and spermine.

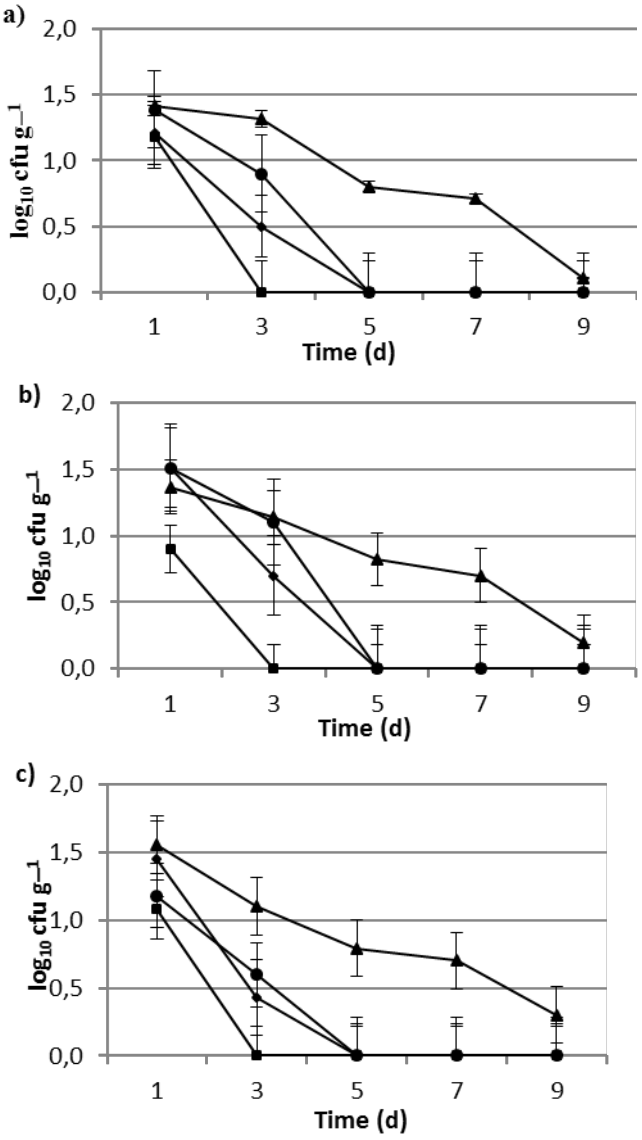


(a) VP, (b) MAP, (c) AP without (▲), and with (♦) DHQ+LA+TH, (■) DHQ+LA and (●) LA

Figure 3. **Variation of the total yeast and molds count in minced pork meat during 9 days of storage at +4 ^{0}C**

Discussions

The hurdle technology, which consists of combining different preservative techniques is an alternative way of preventing growth of pathogens while keeping a high quality (Leistner and Gould, 2012). In this study, we intended to find some combinations of antimicrobial compounds which are able to inhibit the growth of spoilage bacterial and fungal in meat in order to improve quality and shelf-life.

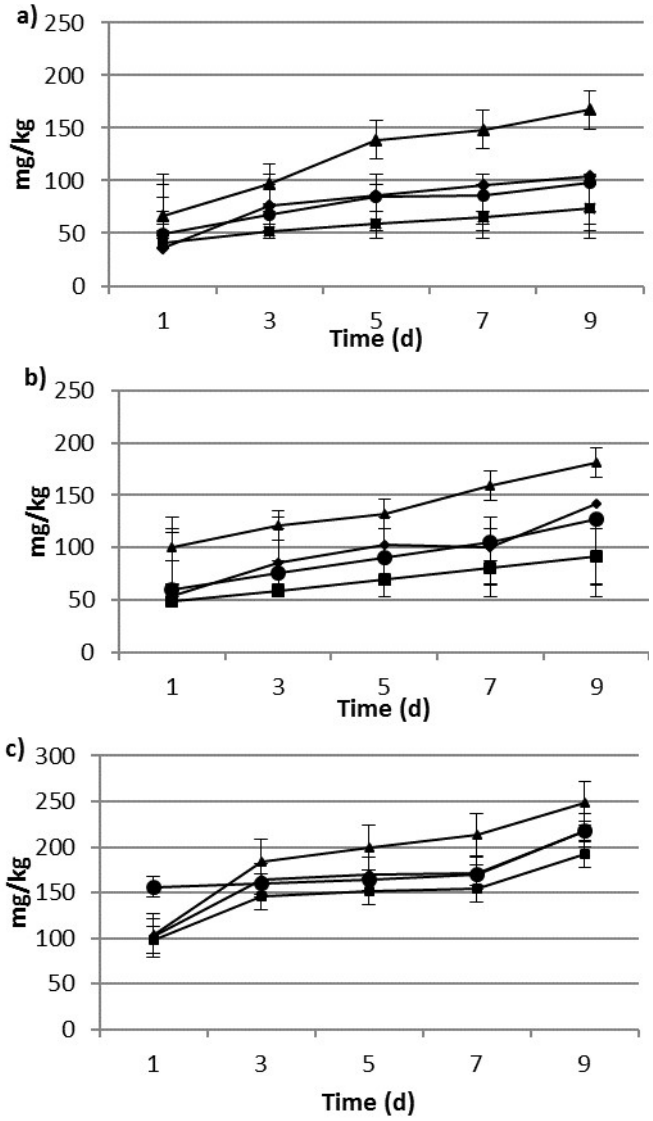


(a) VP, (b) MAP, (c) AP without (▲), and with (♦) DHQ+LA+TH, (■) DHQ+LA and (●) LA

Figure 4. **Variation of the *E. coli* count in minced pork meat during 9 days of storage at +4 ^{0}C**

We determined that LA and its mixtures with DHQ and TH effectively inhibited bacterial growth or reduced numbers of viable bacteria (P<0.05) compared to control sample. In general, the antimicrobial activity of these mixtures increased when the pH decreased. Its difficult to understand the exact mechanism for the establishment of the enhancing antimicrobial effect caused by the combined application of DHQ and TH with organic acids. Research studies on the antibacterial mechanism of phenolic compounds have found damage to cellular membrane changing their structure and function (Dimitriević et al., 2007). Lin and others (2004) suggest that the damage to the cell membrane might explain the

synergic effects, since phenolics as TH could cause sublethal injury to the bacterial cell membrane causing it to become more susceptible to acid environments. Moreover, at low pH the molecules of DHQ and TH are mostly dissociated, more hydrophobic, and bind better to hydrophobic regions of the membrane proteins resulting in better partition into the lipid phase of the bacterial membrane (Hsieh et al., 2001; Gutierrez et al. 2009). Also Dimitriević and others (2007) noted that the antibacterial effect of plant-dervied compounds (*Thymus vulgaris* and *Rosmarinus officinalis*) was noticeably increased using it with LA. The same synergistic effect were reported by Naveena and others (2006), who found that the combination of *Syzygium aromaticum* EO and LA provided a decrease of psychrotrophic and coliform counts in buffalo meat.



(a) VP, (b) MAP, (c) AP without (▲), and with (♦) DHQ+LA+TH, (■) DHQ+LA and (●) LA

Figure 5. **Variation of the total amount of biogenic amines in minced pork meat during 9 days of storage at +4 ^{0}C**

BAs can be produced both by Gram-positive and Gram-negative bacteria (Landete et al., 2008; Marcobal et al., 2012; Wunderlichová et al., 2014). Also some fungi (yeast and molds) are involved in BA accumulation (in particular cadaverine and putrescine), but their role is debated and, for many aspects, controversial (Tristezza et al., 2013; Qi et al., 2014).We find a weak positive correlations between BA contents and total yeast and molds count ($R=0.389$, $P<0.01$). However, correlations between BA contents and ACC and *E. coli* counts were not observed. The capability to form BA is generally considered a strain specific characteristic rather than a species property. Its thus difficult to find precise correlations between BA contents and ACC count (Standarova et al., 2008; Suzzi and Gardini, 2003). Amine production has been recognised as a defense mechanism of microorganisms against an acidic environment (Karovičova and Kohajdova, 2005; Suzzi and Gardini, 2003). Some strains, with amino acid decarboxylase activity, could overcome or reduce the effects of temperature, NaCl, and other biological and chemico–physical factors that induce stress responses in the cells, with the production of some BA (Galcano et al. 2009; Karovičova and Kohajdova, 2005). We find a weak positive correlations between BA contents and pH ($R=0.356$, $P<0.01$). Mah and Hwang (2009) suggest that LA has a positive effect on the amount of BA in meat products.

The main technologies for food preservation based on atmosphere modification are focused on oxygen exclusion. Nevertheless, in such strategy, the principal aim, in relation to BA presence, is not the inactivation of decarboxylase activity but the inhibition of microbial population with decarboxylating properties. In this perspective, the atmosphere used for packaging can affect the qualitative and quantitative formation of BAs. MAP and VP play an important role in the selection of spoilage microorganisms and, particularly, on decarboxylating bacteria (Curiel et al., 2011). The histamine, putrescine and cadaverine content of VP and MAP samples was significantly lower than that of the AP samples during the storage period ($P<0.05$). The production of these BAs was slowed as the CO_2 content increased, indicating that the increasing concentration of CO_2 inhibited the growth of histamine, putrescine and cadaverine producing bacteria. The results are in agreement with those reported by Rodriguez and others (2015) who investigated the effect of CO_2 concentration on the formation of BAs in shredded cooked chicken breast filet packed in MAP. We suggest with Naila and others (2010), that VP inhibits or delays formation of BA more effectively than MAP and AP, through inhibition of BAs forming bacteria or declined enzymatic activity, but the success of inhibition largely depends on the type of microflora.

Conclusions

Using a combination of VP and all three additives (LA, DHQ and TH) resulted in extended shelf life of minced pork meat for up to five days and could be used by meat industry as natural barrier to control the growth of pathogens and natural spoilage microflora. The use of

combinations in meat may be interesting because it leads to the lowering of doses and was found synergy between compounds. However, in the specific case of TH, despite their great potential, their use in food preservation remains limited mainly due to their intense aroma and toxicity problems. In addition, the use of combinations of different food preservation systems, such as the use of different packaging (VP and MAP), could represent another solution to the above mentioned problem. As regards toxicity, the ingestion of high doses of EOs and can induce serious problems. Moreover, more specific ISO standards are also necessary to assess the legal aspects to set out the definition, the general rules for EOs and DHQ use, the requirements for labeling and the maximum levels authorized.

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