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PAPER

BIO-REDUCTION OF ORGANIC POLLUTANTS IN SUGAR CANE FILTER CAKE BY *ANEURINIBACILLUS MIGULANUS*

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ABSTRACT

Filter cake is a major waste residue remaining after refining raw sugar cane juice and it contains organic materials that may contribute to environmental pollution. In this study a common microorganism found in filter cake was isolated and identified according to 16S rRNA sequencing as *Aneurinibacillus migulanus*. This strain was evaluated for its consumption capacity of organic pollutants, leaving a higher concentration of inorganic carbon, e.g. calcium carbonate, in the feeding source. In fact under normal pH and without the addition of water, carbonaceous pollutants were reduced by 33% after 4 weeks of storage at 37°C. This beneficial application of commonly-existing microorganisms not only makes it possible to reduce environmental pollution significantly, but also to increase calcium carbonate content by 4%, a valuable carbon source which is readily extractable.

- Keywords: *Aneurinibacillus migulanus*, calcium carbonate, filter cake, organic pollutants -

INTRODUCTION

Filter cake is a carbon-rich precipitate left behind after sugar cane juice is filtered by rotary vacuum filters during refining. It is a soft, spongy, amorphous and brownish material. Filter cake is rich in calcium carbonate and also contains some organic compounds (about 3.73% of dry weight) (SATISHA and DEVARAJAN, 2007; YALDAV and SOLOMON, 2006; Gupta *et al.*, 2011). These organic materials may cause environmental pollution, generate intense heat (65°C) and foul odors and they take a long time to decompose (SEN and CHANDRA, 2006). Additionally, the presence of organic residues in filter cake may reduce its industrial application as a calcium carbonate source due to its lower purity, reduced shelf life and darker color (CHRISTIDIS *et al.*, 2004). Nonetheless, if these limitations are eliminated, filter cake may be of value as a byproduct that can be used to produce calcium carbonate which in turn has many applications in various food and chemical industries (ASADI, 2007; KESHAVANATH *et al.*, 2006; SHANKARAIAH *et al.*, 2005; YALDAV and SOLOMON, 2006).

Up to now filter cake has been utilized in compost formation (MEUNCHANG *et al.*, 2004) after inoculation with different fungi (KUMAR *et al.*, 2010). It may also be converted into a vermicompost by some species of earth worms (KHWAIRAKPAM and BHARGAVA, 2009; SUTHAR, 2007a; PRAKASH *et al.*, 2010) to reduce the total organic carbon content and increase its content of nutritious minerals such as calcium, potassium and sodium. However, in Iran this waste material is currently left unprocessed resulting in a serious environmental pollution problem.

Currently there is interest in finding a treatment for filter cake that would reduce pollution and increase its utility. MEIER-STAUFFER (1996) isolated thermophilic bacteria by incubating smears of sugar beet juice at approximately 60°C under aerobic conditions on tryptone yeast glucose (TYG) agar. These isolates were characterized by electron microscopy, including freeze-etching and thin sectioning of whole cells, and SDS-PAGE of SDS extracts of the biomass. The organism *A. migulanus* was identified and characterized as an extremophilic, rod-shaped, spore-forming, Gram- and catalase- positive bacterium that grows in alkaline media and converts proteins, fats, and sugars into simple compounds (Barrow and Feltham, 1993). Given these characteristics *A. migulanus* and similar microorganisms may be suitable for filter cake bio-processing in order to reduce its contribution to environmental pollution and increase the quality, purity and extractability of its calcium carbonate. The main objectives of this study were to identify the microorganisms naturally present in sugar cane filter cake, to select the best species that are naturally active in the byproduct, to introduce a cost-effective biotechnological method for reducing sugar cane filter cake pollution, and finally to exploit the microorganism's capacity for production of high-quality calcium carbonate.

MATERIALS AND METHODS

Materials

Filter cake produced from refining raw sugar cane juice was provided by Hekmataneh Sugar Industry (Hamedan, Iran). Nutrient broth, nutrient agar, nitrate reduction broth, Simmons citrate agar, skim milk agar and sulfide indole motility (SIM) media were obtained from Merck (Merck KGaA, Darmstadt, Germany).

Chemical and microbiological analysis of filter cake

The pH levels of filter cake mud samples were determined according to ISO 10390 (1994). Organic carbon and calcium carbonate contents of filter cake mud samples were measured before and after inoculation, using an organic carbon analyzer (BEHAR *et al.*, 2001) and calcimetric method ASTM D4373-02 (2007). Microorganisms that were inhabiting the filter cake samples were isolated by the pour-plate method (KONEMAN *et al.*, 2006) and cultured in nutrient agar media several times in order to achieve pure colonies. Select characteristics of the isolated microorganisms were analyzed by biochemical tests consisting of fermentation of various sugars (e.g. glucose, arabinose, xylose, mannitol and maltose), hydrolysis of macromolecules (e.g. starch, casein, lecithin and gelatin), catalase activity, utilization of Simmons citrate, nitrate reduction and motility (GORDON *et al.*, 1973; RAJOR, 2002; MCKERREL 2004). Gram staining and other morphological features of isolated strains were studied according to LINDH *et al.* (2009) and THIEL (1999). The dynamics of bacterial growth were studied sectrophotometrically by comparing cell growth (absorbance at 600 nm) versus incubation time. The growth curve was detected by the pour-plate method and the optical density was recorded at 600 nm (OD_{600}) using a spectrophotometer (Penuelas-Urquides *et al.*, 2013).

Analysis of 16SrRNA was performed according to the method of KRSEK and WELLINGTON (1999) and MACIEL *et al.* (2009). For cloning and phylogenetic analyses, a region of approximately 1,500 bp from the 16SrRNA gene was amplified using the primers F27 (5′-AGAGTTTGATCMGGCTCAG-3′) and R1492 (5′- GGTTACCTTGTTACGACT-3′; MORA *et al.*, 1998). The sequences were aligned using the Chimera Check program of the Ribosomal Database Project. All sequences were validated with those available in a public database (Ribosomal Database Project release 9.43; JONGSIK *et al.*, 2007), and only those

with high fidelity were included in the analysis (ALTSCHUL *et al.*, 1997). Phylogenetic trees were constructed using the neighbor-joining method in the MEGA version 4.0 software package (TAMURA *et al.*, 2007).

Preparation of inoculated filter cake specimens

The selected strains were inoculated at a density of 6×10^8 colony forming units (CFU) in the filter cake samples according to the standard turbidity method of McFarland (VOIGHT, 2011). Specimens were prepared at pH 7.5 as a control and also at pH 9.5, alkalinized by 2 M sodium hydroxide solution which diluted by water at 0, 25, 50 or 75%. These preparations were stored for 2 and 4 weeks at 37°C for further analysis. X-ray diffraction was used to analyze the minerals present in the filter cakes after storage for 4 weeks (HARRIS and WHITE, 2008).

Results and Discussion

Chemical analysis of the filter cake specimens indicated that the initial pH was 7.5. The composition was estimated to be 3.72% organic compounds and 84.40% calcium carbonate on a dry weight basis.

Fourteen bacterial strains were isolated from the filter cakes. One strain, A_6, was selected because it formed colonies in the shortest time (8-12 h) at pH 7.5 and 37°C, which are the best conditions for filter cake storage. Because storage conditions and time are both important economic factors to consider in the industry strain A_6 was the ideal candidate.

The results of the biochemical tests performed on strain A_6 are shown in Table 1. Morphological analysis revealed that the purified colonies had a rough, conical or volcano-like appearance, and were wrinkled or mucoid. The colonies were often white, creamy yellow, or had a milky color with smooth, serrated edges (Fig. 1). According to the Bergey's Manual of Systematic Bacteriology by VOS *et al.* (2009), the isolated strain was bacilli and its growth curve is shown in Fig. 2. Examination of the 16S rRNA sequence with Blast software showed that with 99.90% probability the isolated strain was *Aneurinibacillus migulanus* (Fig. 3). As this strain was naturally present in

Table1 - Phenotypic characterization of bacterial culture A_6 isolated from filter cake samples.

Characteristic	A_6
Gram Reaction	+
Nitrate Reduction	+
Casein Hydrolysis	–
Lecithin Hydrolysis	+
Gelatin Hydrolysis	+
Starch Hydrolysis	–
Glucose Fermentation	+
Arabinose Fermentation	+
Xylose Fermentation	+
Mannitol Fermentation	+
Maltose Fermentation	+
Simmons Citrate Utilization	–
Motility	–
Catalase Reaction	+

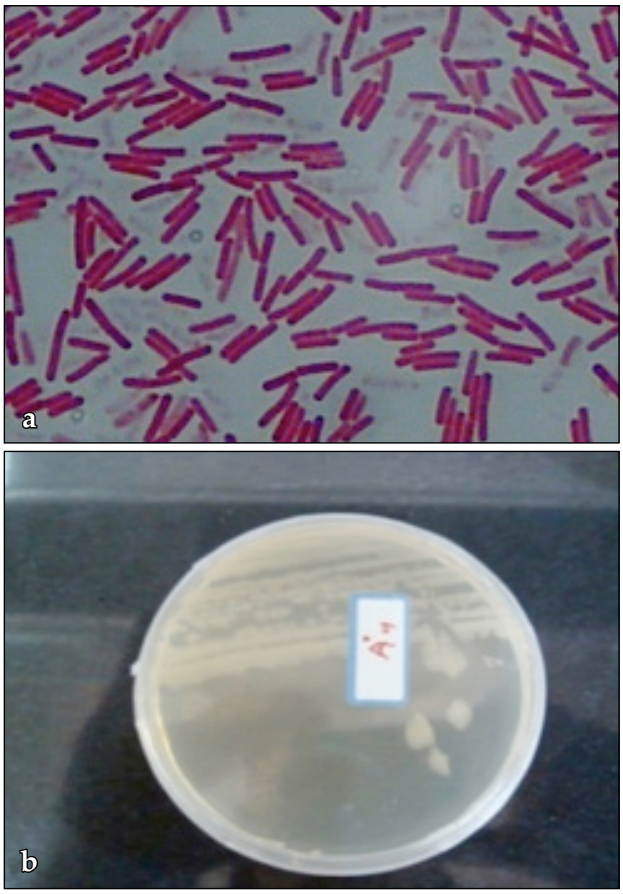


Fig. 1 - Pure colonies of bacterium A_6 isolated from filter cake.

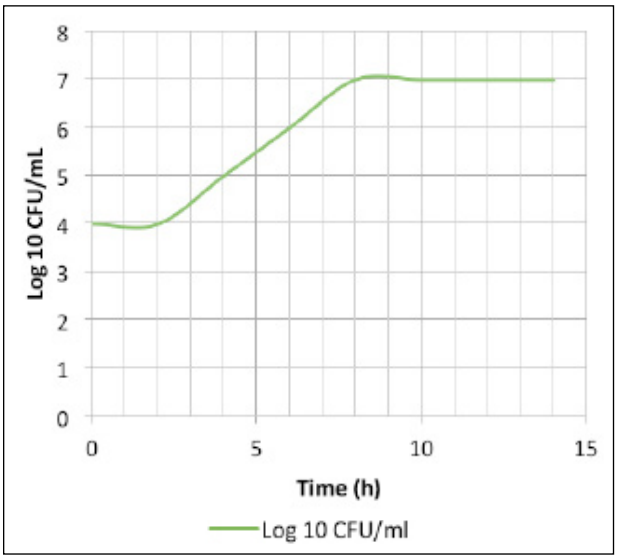


Fig. 2 - Growth rate curve of bacterial isolate A_6 (*A. migulanus*).

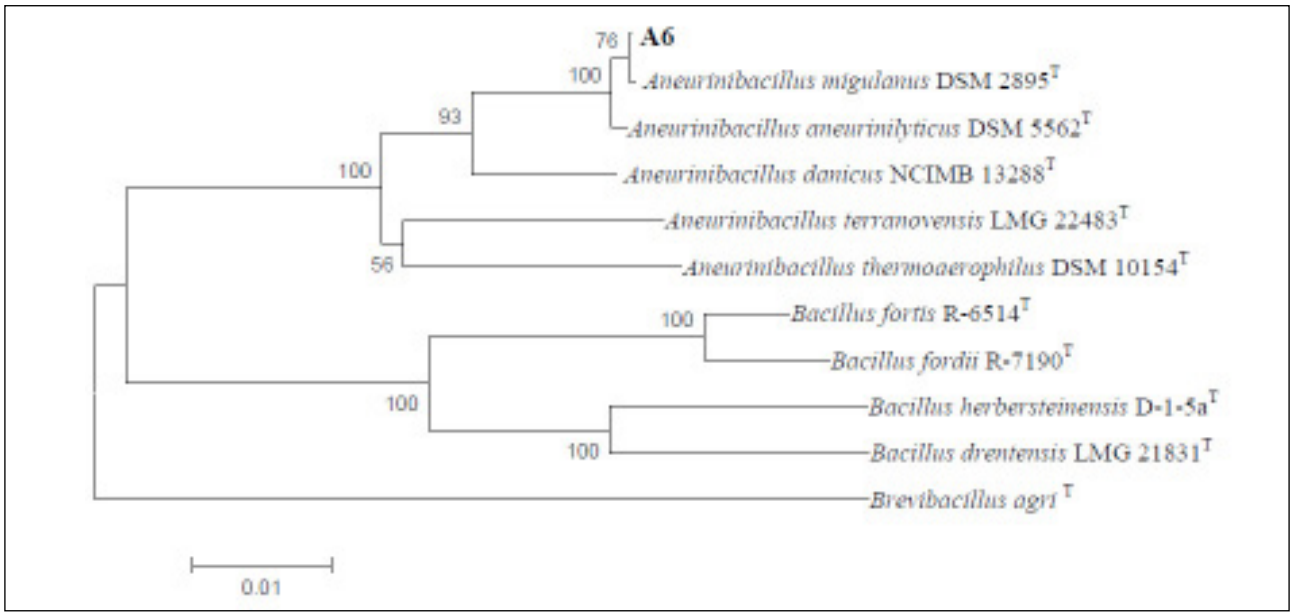


Fig. 3 - Neighbor-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between strain A_6 and the related genera.

filter cake it could tolerate the thermal (50°-80°C) and chemical (relatively alkaline) conditions of this medium during processing so it is assumed to be an extremophile bacterium and can be grouped in thermopiles. TAKAGI *et al.* (1993) and SHIDA *et al.* (1994) investigated the phenotypic and genotypic characteristics of *A. migulanus* and reported similar results.

Changes in the contents of organic compounds were computed by Analysis of Variance. The reduction in organic compound content in filter cake specimens after inoculation with *A. migulanus* was in the range of 19% to 52% from its initial level of 3.72% (Table 2). However the results of the Analysis of Variance revealed that none of the variables (i.e., time, concentration, pH nor any combination of these variables) had a statistically significant influence on carbon content. The average reduction among the different treatments was 33.80%. According to a study by MEIER-STAUFFER (1996) this strain may only be able to break down simple compounds like fructose and non-complex polysaccharides, so this may limit its consumption or-

Table 2 - Contents of organic compounds and $CaCO_3$ at various dilutions, pH levels and storage time periods.

Filter cake treatment parameters				
pH	**Dilution (%)**	**Storage time (weeks)**	**Organic compound content (%)**	**Calcium carbonate content (%)**
7.5	0	2	2.80±0.02	84.17±4.57
		4	2.49±0.42	87.74±1.19
	25	2	2.49±0.14	81.62±4.21
		4	2.35±0.10	88.53±0.73
	50	2	2.41±.26	87.72±1.49
		4	1.77±0.33	86.89±1.44
	75	2	2.53±0.73	85.21±3.11
		4	2.13±0.13	86.75±0.22
9.5	0	2	2.26±0.22	86.35±0.45
		4	1.68±0.22	88.85±0.48
	25	2	2.25±0.21	87.05±0.96
		4	1.86±0.70	83.35±7.34
	50	2	2.48±1.14	85.24±4.13
		4	2.37±0.38	86.21±0.19
	75	2	2.45±0.72	86.16±1.41
		4	2.19±0.01	85.96±0.16

Data are presented as means ± standard deviations, reported on a dry weight basis.

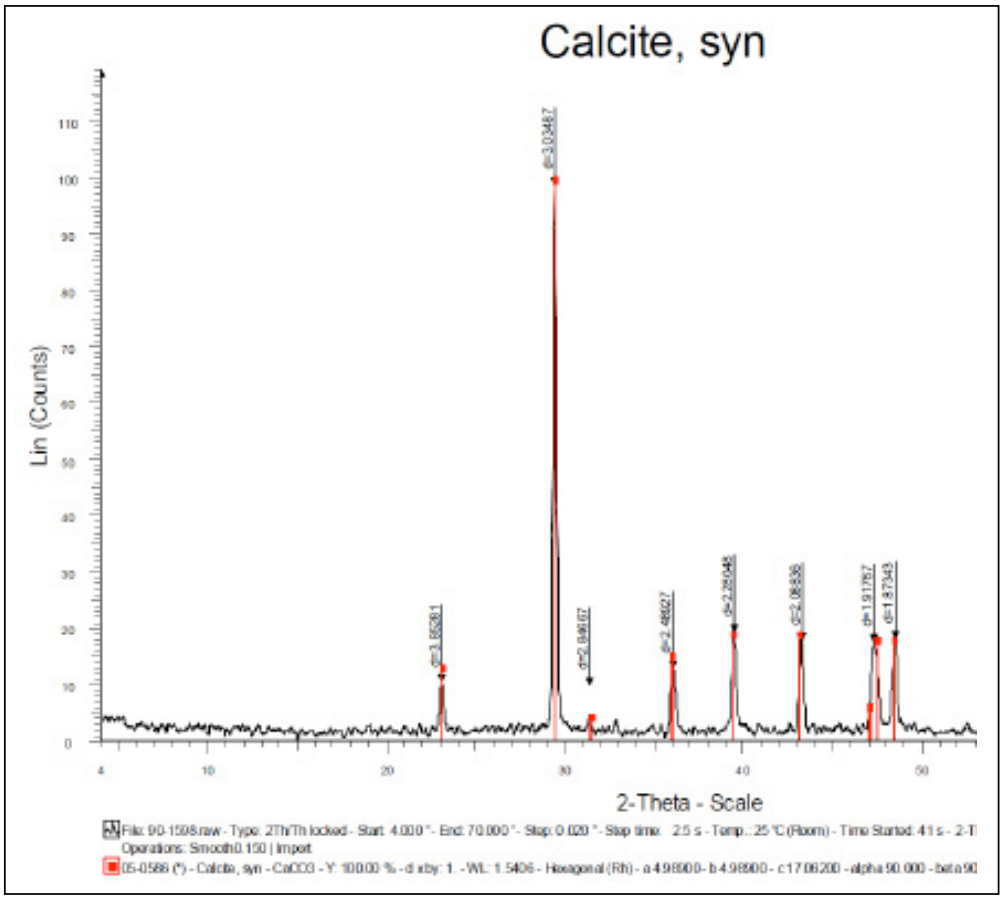


Fig. 4 - X-ray diffraction pattern for processed filter cake after 4 weeks of storage.

ganic compounds in the current study as well. On the other hand the current results are better than have been reported elsewhere; microbial composts and vermicomposts prepared by KHWAIRKPAM and BHARGAVA (2009) and KUMAR *et al.* (2010) only reduced total carbon content by about 4-15%. In comparison the microorganisms naturally present in filter cake used in our research performed with higher efficiency at lower cost.

Changes in the calcium carbonate content were analyzed by Analysis of Variance. The results in Table 2 show that calcium carbonate content changed from 84.42 to 86.11%, with an average increase of 4%. Again the Analysis of Variance revealed that none of the variables (i.e., time, concentration, pH nor any combination of these variables) had a statistically significant impact on carbon content. Regardless it is possible that the purity of the calcium carbonate in the filter cake was increased by the microbial elimination of organic compounds.

The X-Ray diffraction pattern for the processed filter cake after 4 weeks of storage is shown in Fig. 4. In this pattern the highest intensity peak is attributed to calcite (calcium carbonate) with a *d*-spacing of approximately 3.04 (HARRIS and WHITE, 2008).

CONCLUSIONS

The results from this study suggest that bioreduction of organic carbonaceous pollutants in sugar cane filter cake are feasible with the assistance of microbes naturally occurring in the byproduct. No additional treatment was necessary and only storage at its natural pH would be sufficient to provide a suitable condition for the growth of *Aneurinibacillus migulanus*, one of the microorganisms present in filter cake. By this cost effective method the organic pollutants were microbially reduced and the filter cake, which is usually considered an environmentally-problematic waste, was converted to a valuable source of minerals, especially calcium carbonate.

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PAPER

QUALITATIVE AND SENSORY EVALUATION OF SANGIOVESE RED WINE OBTAINED FROM ORGANICALLY AND CONVENTIONALLY GROWN GRAPES

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ABSTRACT

This study compared organic and conventional Sangiovese PDO (Protected Denomination of Origin) wines from two successive vintages using sensory descriptors and instrumental parameters. Organic and conventional wines differed for almost all instrumental parameters, whereas sensory differences were less pronounced. Wines from different vintages were clearly distinguished. Differences related to agricultural techniques were found for vintage 2007, organic wines showing stronger sour taste and astringent sensation as well as higher values of total polyphenols, and total and free anthocyans than conventional wines. Conventional and organic wines from vintage 2008 differed only for phenolic compounds and astringent sensation, which were higher in the organic wines.

- Keywords: organic wine, phenolic compounds, sensory analysis, sustainable agriculture -

INTRODUCTION

In terms of wine sector economy, understanding consumer's food habits is the key point for guiding institutional and managerial policies. When consumers make choices about wine, they are concerned with an enormous amount of information such as grape variety, brand label, grocery retailer indications, production methods and information about sustainable or 'green' wine practices (GOODMAN, 2009; LOCKSHIN and CORSI, 2012).

As regards wine sustainability, consumers have become increasingly concerned by the effects of conventional agricultural production practices on both human and environmental health. As a consequence, produce obtained from organic farming methods has been rapidly increasing in developed countries. Indeed, although the economic crisis has recently had a major impact on food trade, a reverse trend has been observed for organically grown products market, which increased of 7.3% in large scale retail trade (NOMISMA, 2013). This may be explained by the fact that organic food adequately meets all requirements for quality, genuineness and healthiness (FORBES *et al.*, 2009).

While organic wine still represents a market niche, it has increased considerably in the last few years also due to the recent Commission Regulation (EC) No 203/2012, which entered the market and made its way in stockists' shelves in 2012 with full organic labelling, including the European organic logo. Before this regulation, only "*wine from organic grapes*" could be sold, but "*organic wine*" could not. According to the new organic wine rules, growers can now use the term "*organic wine*" on their labels (GAETA and CORSINOVI, 2014). However, practices and techniques for the production of wine are established at the Single Common Market Organization level (Reg. 1308/2013).

According to Nomisma data (NOMISMA, 2013), the total Italian surface devoted to organically grown grapes corresponds to 53,000 ha. The export sales seem to be promising for Italy, since 27% of the wines sold in US in 2012 were imported with a prevalence of Australian (35%) and Italian (25%) products. In this context, if we consider Italian organic wines, the percentage corresponds to 13%. This percentage is likely to be increased by the recent US EU Organic Equivalence Arrangement, which allows since June 2012 the trade of Italian wine from organically grown grapes without the need of a second American certification.

The interest in organic food by consumers has also led to an increase of scientific literature on this topic, even though studies are still scarce. Studies comparing foods derived from organic and conventional growing systems focused mainly on three topics: nutritional value, sensory quality and food safety.

Relative to the nutritional value of wine, attention was mainly directed toward its antioxidant activity and consequent benefit on health. Research has shown that moderate red wine consumption can reduce the risk of cardiovascular disease (RENAUD and DE LORGERIL, 1992). The protective effects of wine have been attributed to phenolic compounds that are efficient scavengers of free radicals and breakers of lipid peroxidative chain reactions. Besides antioxidant activity, phenols also have anti-inflammatory effects and may protect low-density lipoproteins (LDL) against oxidative modification (AKÇAY *et al.*, 2004). Several studies have been performed to compare phenolic compounds content and antioxidant activity of organic and conventional wines but contradicting results were found (MULERO *et al.*, 2010; VRČEK *et al.*, 2011; ZAFRILLA *et al.*, 2003).

In terms of the sensory quality of food products, reports indicate that organic and conventional fruit and vegetables may differ on a variety of sensory aspects (BOURN and PRESCOTT, 2002). Generally speaking, there are two opposite opinions regarding sensory properties of organic and conventional food. On one hand, organically grown fruit and vegetables, and thus grapes, being not subject to pesticides that protect against pest, may be more susceptible to microbiological contamination than conventional grapes, thus resulting in products with lower sensory quality. On the other hand, organic methods can potentially produce fruit and vegetables with better taste and flavor due, for instance, to low NPK fertilization, which results in low yields and a high concentration of sugars, total dissolved solids and dry matter (BASKER, 1992; HAGLUND *et al.*, 1999). Nevertheless, literature data failed to find consistent differences between organic and conventional products from both a hedonic and sensory point of view (BOURN and PRESCOTT, 2002). Therefore, the assumption of organic food having a better taste may be explained by the consumer's expectation of a healthier and safer product evoked by the label "organic food" (DELIZA and MACFIE, 1996).

Very few studies have been addressed to the comparison of sensory properties of wines derived from organically and conventionally grown grapes. MOYANO *et al.* (2009) compared the aroma profile of sherry wines cultivated conventionally and organically and found that the organically aged wines had a sensory profile similar to that of the conventional one, but lower odor intensity. The same findings were reported by DUPIN *et al.* (2000), who found that organic products tended to be less aromatic than conventional ones.

'Sangiovese' grape variety (*Vitis vinifera L.*) is currently grown in Italy on about 69,000 ha. It is the most widely used Italian vine variety and is included in the ampelographic description of a number of Protected Denomination of Origin

(PDO) wines, among which prestigious Tuscan wines such as Chianti and Brunello di Montalcino. Nevertheless, Sangiovese is cultivated in regions other than Tuscany (*e.g.* Emilia Romagna and Umbria) (PONI *et al.*, 2007). To the knowledge of the present authors, no studies are available on Sangiovese red wine sensory and nutritional quality despite being largely known and consumed worldwide. Thus, the aim of this work was to compare organically and traditionally grown Romagna Sangiovese PDO red wines (Gazzetta Ufficiale Italiana, 2011) from two different vintages. In order to compare the wines, sensory descriptors and instrumental parameters were taken into account.

MATERIALS AND METHODS

Wines

Red wines evaluated in the present study were produced from ripe grapes from *Vitis Vinifera* var. Sangiovese harvested in September 2007 and 2008 in Faenza, situated in the eastern part of the Emilia Romagna region (Italy). Grapes derived from two different farms located in adjacent areas subjected to similar environmental conditions. One farm produced grapes according to organic techniques, whereas the other adopted conventional agricultural techniques. Organic management used cover crops of native wild species with spontaneous selection and regular addition of organic matter (manure and compost). In addition, neither insecticides nor synthetic fertilizers were used in organic agriculture during the crop. Conversely, conventional agriculture used fertilizers and pesticides to control weeds, pests and diseases; these chemicals are subject to rigorous testing and authorization procedures before they can be used, and winegrowers have respected the guidelines and restrictions to use them. Grapes of the two different vineyards were produced by using the same growing practices (*e.g.* same trellis).

Chemico-physical and sensory comparison was performed on a total of 4 different wines (organic wine 2007 *vs* conventional wine 2007; organic wine 2008 *vs* conventional wine 2008). All wines were produced following the same process. Winemaking conditions consisted of the addition of 10 mg/L of SO_2 after stemming and crushing, followed by fermentation (size of fermentation 200 L) in stainless steel tanks with the inoculation of selected yeasts (Zymaflore F15, Laffort); temperature was maintained at 29°C. Manual punching down was done twice a day, and separation of wine from solids was performed when relative density reached a constant value. Subsequently, wine was racked five times; during the last three racking phases, organic and conventional wines were added with respectively 10 mg/L and 20 mg/L of SO_2. The malolactic fermentation was induced by inoculation with *Oenococcus oeni* lactic acid bacteria (Viniflora CH35, Laffort). Wines were cold stabilized, added with 15 mg/L of SO_2, and then bottled and stored in a room with a constant temperature between 16° and 18°C. Three bottles from organic and three for traditional production for each vintage were randomly selected to be used for chemico-physical analyses. A further twelve bottles for each vintage selected in the same way underwent sensory analysis.

For each vintage, chemico-physical and sensory analyses were performed three months after wines were bottled and thus when analyzed, wines had the same age.

Chemico-physical analysis

pH was determined with a model 62 pH meter (Radiometer Copenhagen, Denmark); total acidity (expressed as g/L of tartaric acid,), volatile acidity (expressed as g/L of acetic acid) and pH were evaluated following the procedures of OIV (Office International de la Vigne et du vin, 1990); total polyphenols (mg/L of gallic acid), total anthocyans (mg/L of malvidin monoglucoside), free anthocyans (mg/L of malvidin monoglucoside), total flavonoids (mg of (+) catechin) and non-anthocyan flavonoids (mg of (+) catechin) content were measured as described by Di Stefano and Cravero (2001), using a Hewlett-Packard 8453 spectrophotometer (Hewlett-Packard, Milano, Italy). All chemico-physical analyses were performed in triplicate.

Sensory analysis

Descriptive analysis (LAWLESS and HEYMANN, 1998; ISO 13299, 2003) was used to identify and quantify the sensory properties of organic and conventional wines from successive vintages.

Sixteen assessors (11 women and 5 men from 23 to 35 years of age) were selected from a panelist pool consisting of University of Milan students and employees, who reported liking red wine and consuming it more than twice a month. None of the participants had previous or present taste or smell disorders.

Subjects were trained over a period of two months (eight 1-h training sessions). In a preliminary session, the goals of the experiment were explained in detail to participants (*i.e.* to perform a description of red wines; differences in agricultural techniques between wines were not mentioned). During the training period, assessors attended a number of group sessions in which they tasted the wines and generated twelve descriptors: 4 odor descriptors (fruity, spicy, woody and vanilla), 2 taste descriptors (sour and bitter), 3 flavor descriptors (fruity, spicy and woody) and 3 mouthfeel sensations (astringent, alcohol and body). Each descriptor was extensively discussed and explained to avoid any confu-

sion about the relevant meaning. Once the vocabulary was set up, assessors performed four sessions in sensory booths to acquire familiarity with the scale. In order to test panel repeatability, one wine was presented twice in the samples series as a hidden control.

After training was completed, for each vintage and in each tasting session, the panel evaluated two wines (organic *vs* conventional) in a balanced order (half assessors tested the organic wine first and the other half of assessors tasted the conventional wine first). The tasting of the same two wines was repeated three times on the same day (with a minimum of 2 hours-break between the sessions and an interval of minimum 2 hours between replicates). Judges were instructed to drink and swallow each sample and rate the intensity of all attributes of the first wine using a 9-point category scale ranging from 1 (absence of the sensation) to 9 (maximum intensity) and then to move to the second wine. Assessors were asked not to smoke, eat or drink, except water, for one hour before the tasting sessions. For each sample, judges received a 30 mL sample served in ISO glasses (ISO 3591, 1977) coded with a 3-digit number and covered with a Petri dish to avoid the escape of volatile components. Participants were provided with mineral water and unsalted crackers to clean their mouth between the tasting of each wine. Wines were served at 18 ± 1 °C. In order to balance the effects of serving order and carryover, presentation orders were produced according to William Latin squares (MACFIE *et al.*, 1989). Wines from vintage 2008 were evaluated one year later with the same procedure and by using the same panel that was kept trained over time. The study was performed in accordance with the Declaration of Helsinki. The protocol was approved by the Institutional Ethics Committee at the study site. Informed consent was obtained from all subjects. All sensory sessions were conducted at the sensory laboratory of the Department of Food, Environmental and Nutritional Sciences (DeFENS, Università degli Studi di Milano) designed in accordance with ISO guidelines (ISO 8589, 2007). Data acquisition was done using Fizz v2.31 software (Biosystèmes, Couternon, France).

Data analysis

For each vintage, chemico-physical data were submitted to *t*-test ($p<0.05$) in order to compare the two methods of production for each parameter considered.

For each vintage, sensory data were analyzed by means of Analysis of Variance (ANOVA) considering *Wines* (organic *vs* conventional), *Judges*, *Replicates* (rep 1 *vs* rep 2 *vs* rep3) and the interactions *Judges x Wines*, *Judges x Replicates* and *Wines x Replicates* as factors. The factor *Judges* was considered random in the model. When the ANOVA showed a significant effect ($p<0.05$), the Least Significant Difference (LSD) was applied as multiple comparison test.

Sensory and chemico-physical data were then jointly elaborated by Principal Component Analysis (PCA) in order to examine the results from a multi-dimensional point of view. For each vintage, sensory data were averaged across judges, whereas for chemico-physical data from each of the three replicates were used. Data were centered and auto-scaled prior to any modeling. This preprocessing technique is required when variables showing different variation ranges need to be compared. It gives all variables the same chance to influence the estimation of the components. PCA was performed on correlation matrix. Cross validation was applied as a validation method. A preliminary PCA was made considering all variables: correlation loading plot was performed and variables with less than 50% explained variance were left out in further analyses (WESTAD *et al.*, 2003). Data were treated using SAS/STAT statistical software package version 9.1.3. (SAS Institute Inc., Cary, USA) and The Unscrambler® 9.8 statistical software (Camo As, Trodheim, Norway).

RESULTS

Chemico-physical analysis

Chemico-physical results are reported in Table 1. Considering vintage 2007, wines differed for all parameters with the exception of non-anthocyanin flavonoids and total flavonoids. Organic wine showed a higher value of total acidity and consequently a reduced pH value as compared to the conventional one. It also had a higher content of total polyphenols, total and free anthocyans and a lower volatile acidity value. Differences between the two wines were also found for vintage 2008 for all parameters except for pH, total and free anthocyans. Organic wine had lower volatile and total acidity values and higher contents of non-anthocyanin flavonoids, total flavonoids and polyphenols than the conventional wine.

Sensory analysis

Mean intensity ratings of organic and conventional wines are reported in Tables 2 and 3. Relative to vintage 2007, wines differed mainly for sour taste ($p<0.01$), bitter taste ($p<0.05$) and astringency ($p<0.001$). According to post-hoc comparison test, the organic wine was perceived with a higher intensity of sour taste and astringent sensation. Conversely, the conventional wine had a more pronounced bitterness. Wines from vintage 2008 differed only for astringency ($p<0.01$), with organic wine having a higher intensity for such descriptor.

Table 1 - Chemico-physical results: Comparison between organic (Org.) and conventional (Conv.) wines for each chemico-physical parameter for both vintages. Values are reported as mean values of triplicates (± SEM).

Chemico-physical parameters	Vintage 2007			Vintage 2008		
	Org.	Conv.	*p-value*[(a)]	Org.	Conv.	*p-value*[(a)]
pH	3.00±0.02	3.39±0.02	*<.0001*	3.51±0.02	3.48±0.02	*n.s.*
Volatile acidity (g/L acetic acid)	0.35±0.01	0.55±0.01	*<.001*	0.30±0.01	0.36±0.01	*<.05*
Total acidity (g/L tartaric acid)	6.42±0.01	5.02±0.01	*<.0001*	6.32±0.01	6.75±0.01	*<.0001*
Non-anthocyanin flavonoids (mg/L (+) catechin)	2410±48	2465±48	*n.s.*	1481±48	1222±48	*<.001*
Total flavonoids (mg/L (+)catechin)	2800±48	2713±48	*n.s.*	1893±48	1636±48	*<.001*
Total polyphenols (mg/L gallic acid)	2687±13	2180±13	*<.0001*	1712±13	1168±13	*<.0001*
Total anthocyans (mg/L malvidin)	270±2	172±2	*<.0001*	283±2	284±2	*n.s.*
Free anthocyans (mg/L malvidin)	96±3	48±3	*<.01*	170±3	173±3	*n.s.*

[(a)] *p*-value as obtained by *t*-test comparison.

ANOVA results showed a significant effect ($p<0.001$) of the factor *Judges* for all attributes the effect, which is expected because individuals may have their own frame of reference, despite the attempt to decrease this gap during training sessions (LEA *et al.*, 1997). *F*-values for *Replicates* and interactions between *Wines x Judges*, *Judges x Replicates* and *Wines x Replicates* were not significant for nearly all the attributes. This means that there was no systematic difference from one replicate to another and that judges were repeatable.

Relationship between sensory and chemico-physical data

In order to obtain an exhaustive characterization and differentiation of the wines according to both method of production and vintage, data obtained by chemico-physical and sensory evaluations were jointly elaborated by PCA, considering the two types of wines (organic *vs* conventional) for both vintages (2007 *vs* 2008). The score and loading plots in the plane defined by PC1 and PC2 (84% total variance explained) are shown in Figs. 1 and 2, respectively. In the score plot (Fig. 1), a clear separation between products can be observed. PC1 (53% explained variance) mainly differentiates products according to vintage, with wines from vintage 2007 and 2008 positioned in the negative and positive part of PC1, respectively. PC2, explaining a further 31% of total variance, separated wines from vintage 2007 according to the method of production, with organic and conventional products located in the positive and negative part of PC2, respectively.

From Fig. 2 it can be observed that, in the negative part of PC1, the sensory descriptors woody flavor, alcohol, astringent and body are positioned near to total polyphenols, total flavonoids and non-anthocyanin flavonoids con-

Table 2 - Sensory analysis results: mean intensity values for each sensory descriptor by method of production for vintage 2007. For each descriptor the relevant significance is reported (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). Significant effects are reported in bold.

Descriptors	Vintage 2007	
	Organic	Conventional
Fruity aroma (n.s.)	5.5	5.2
Spicy aroma (n.s.)	4.5	4.9
Vanilla aroma (n.s.)	4.7	5.1
Woody aroma (n.s.)	4.0	4.4
Sour (**)	**5.7**	**4.7**
Bitter (*)	**4.4**	**5.2**
Fruity flavor (n.s.)	4.5	4.3
Spicy flavor (n.s.)	4.2	4.3
Woody flavor (n.s.)	3.9	3.4
Astringent (***)	**6.2**	**4.6**
Alcohol (n.s.)	5.9	5.5
Body (n.s.)	5.7	5.4

Table 3 - Sensory analysis results: mean intensity values for each sensory descriptor by method of production for vintage 2008. For each descriptor the relevant significance is reported (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). Significant effects are reported in bold.

Descriptors	Vintage 2008	
	Organic	Conventional
Fruity aroma (n.s.)	5.1	5.1
Spicy aroma (n.s.)	4.1	4.3
Vanilla aroma (n.s.)	4.9	4.8
Woody aroma (n.s.)	3.7	3.7
Sour (n.s.)	5.2	5.3
Bitter (n.s.)	4.5	4.3
Fruity flavor (n.s.)	4.6	4.9
Spicy flavor (n.s.)	4.1	4.2
Woody flavor (n.s.)	3.1	3.0
Astringent (**)	**5.1**	**3.9**
Alcohol (n.s.)	5.3	5.1
Body (n.s.)	5.0	4.8

tent. These variables are opposed to total and free anthocyans content, which are in turn positioned near to fruity flavor. On PC2 (positive part), sour taste is positively correlated to the relevant chemico-physical parameter and opposed to bitter taste, woody aroma, spicy aroma and volatile acidity. Comparing Figs. 1 and 2, it can be seen that organic and conventional wines from vintage 2008 are very close to each other on the positive axis of PC1 and are characterized by high pH values and a high content of free and total anthocyans and by reduced total polyphenols, total flavonoids and non-anthocyan flavonoids content. These wines are mainly described by fruity flavor and showed low intensity of spicy and woody aromas as well as a reduced body, astringency and alcohol sensations. Considering wines from vintage 2007, organic wines differed from conventional wines because they were perceived as more astringent, alcoholic and with more pronounced body and woody flavor. Coherently, they showed a higher content of total polyphenols, flavonoids and non-anthocyan flavonoids. In addition, organic wines were described by low pH values. Conversely, conventional wines from vintage 2007 were mainly described by bitter taste, woody and spicy aromas and high values of volatile acidity.

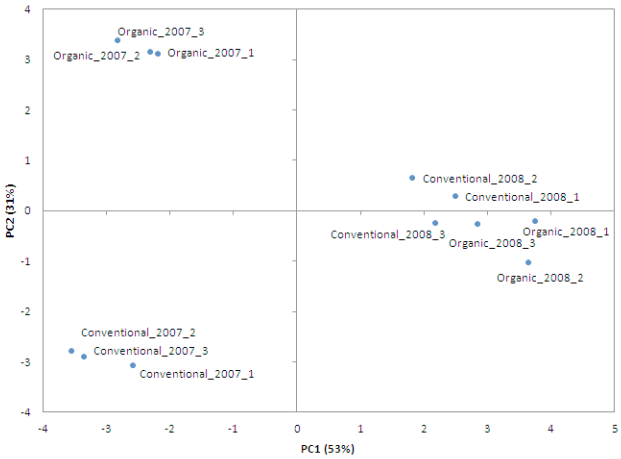


Fig. 1 - Score plot obtained by the PCA model of organic and conventional wines from both vintages considering sensory and chemico-physical data (numbers from 1 to 3 refer to replicates, ar =aroma, fl = flavor).

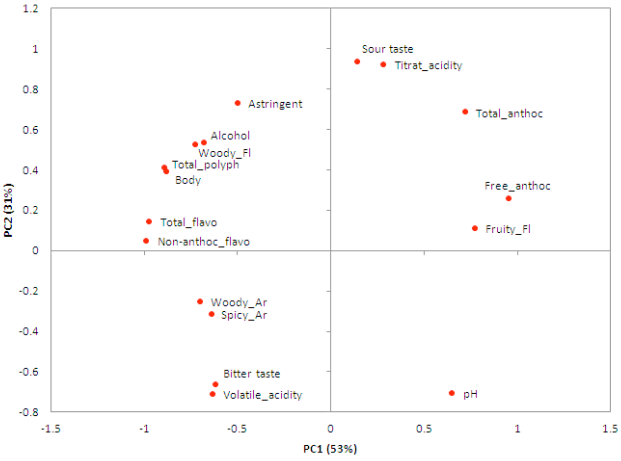


Fig. 2 - Loading Plot obtained by the PCA model of organic and conventional wines from both vintages considering sensory and chemico-physical data (numbers from 1 to 3 refer to replicates, ar =aroma, fl = flavor).

DISCUSSION

The present study compared Romagna Sangiovese PDO red wine deriving from organically and conventionally grown grapes of two successive vintages. One of the main findings of the study is that organic and conventional wines differed from an analytical point of view, in particular organic wines showed higher phenolic compounds compared to the conventional ones in both vintages. Sensory-related differences were less pronounced and concerned mainly taste (sour and bitter) and mouthfeel sensations (astringent).

There are fundamental differences in organic and conventional production practices that may in part explain this result. Organic systems emphasize the accumulation of organic matter over time through the use of cover crops, manure, and composts. Conventional farms utilize fertilizers containing soluble inorganic nitrogen and other nutrients, which are more directly available to plants, thus influencing the synthesis of secondary plant metabolites, proteins, and soluble solids (RAPISARDA *et al.*, 2005). Also, organically produced plants have a longer ripening period compared to conventional plants because of a slower release of the supplied nutrients (BRANDT and MØLGAARD, 2001). As secondary plant metabolites like polyphenols are formed in the ripening period, one could expect a higher content of these compounds in organically grown plants. However, there is a difference of opinion about the nutritional quality of organic and conventional wines presented in literature. For instance, in agreement with the present study, higher phenol content and antioxidant activity were detected in organic wines compared to conventional wines by VRČEK *et al.* (2011). MULERO *et al.* (2010), found that phenolic compounds and antioxidant activity were slightly higher in organic wine than in conventional wine, although the differences were not significant. Conversely, ZAFRILLA *et al.* (2003) did not find differences in the antioxidant activity between organic and conventional red wines but differences were detected for white wines. Of course, this disagreement of data could be attributed to several variables, such as vine, area of origin and vintage. Thus, the question whether organic and conventional wines differ in their nutritional quality is still an issue to pursue.

The differences between organic and conventional wines found from an analytical point of view, seem to have had little impact on sensory perception. Indeed, the results of the present study indicate that sensory-related differences were marginal and concerned mainly taste and mouthfeel sensations. Organic wines from vintage 2007 were perceived as more sour, astringent and less bitter than conventional wines, whereas differences for vintage 2008 concerned only astringency. These results are coherent with the pattern observed for chemico-physical analyses showing that for both vintages the organic wines showed higher values of total polyphenols, which are mainly responsible for the perception of astringency. In the same way, the organic wine from vintage 2007 had a higher level of total acidity than its conventional counterpart, thus resulting in a product with a high perceived sourness. The sensory comparison of wines deriving from organic and conventional agriculture is interesting due to the lack of information on this specific issue. It seems that studies comparing organic and conventional wines from a sensory point of view did not find differences in the sensory profile of the products, even though organic wines tended to be less aromatic (MOYANO *et al.*, 2009; DUPIN *et al.*, 2000). As data available on sensory-related differences in organic and conventional wines are too few in number to draw general conclusions, further research is needed to clarify this aspect.

A good correlation between sensory and chemico-physical measurements was found. Sour taste was positioned near the relevant chemico-physical parameter total acidity, whereas astringency was located near the phenolic compounds. As previously mentioned, these results are coherent with literature data reporting that astringency is a complex sensation inducing a dry, pucker, rough sensation typically found in red wines (Lawless *et al.*, 1994) induced by phenolic compounds, which bind with salivary proteins and glycoproteins causing their precipitation. The polyphenols in red wines believed to be most responsible for astringency are the polymeric flavan-3-ols, which occur either as galloylated (esterified with gallic acid) species, conjugated with anthocyanins, or in the free form. The flavan-3-ols derive primarily from the seeds and skin of the grape and are extracted during fermentation (JACKSON, 2002). In addition, in the PCA model, astringency was opposite to pH; this is in line with data reporting that pH is an important factor influencing the perception of astringency in wines. More specifically, the hydrogen ion concentration affects protein hydration, and both phenol and protein ionization. In fact, in excessively acidic wines, the low pH can independently induce sufficient salivary glycoprotein precipitation to elicit the sensation of astringency (GUINARD *et al.*, 1986).

In conclusion, the present study evidenced differences between Sangiovese red wines derived from organic and conventionally grown grapes. The main differences were related to the chemico-physical pattern. A limitation of this study is that only two vintages of one grape variety of organic and conventional wines were considered. Therefore, it is advisable to extend the study to other grape varieties considering a broader range of vintages in order to clarify whether organic and conventional wines show a different sensory and nutritional quality. This aspect seems to be particularly relevant since environmentally sustainable practices related to wine quality seem to have good market prospects.

Future perspectives of the study will be focused on the evaluation of consumer's willingness to pay for organic and conventional PDO wines and the investigation of how the consumer's behavior can be influenced by no-prices factors such as the nutritional information on label, which will become obligatory throughout Europe from 2016 on in accordance with Horizontal Regulation 1169/2011.

All authors certify that there are no affiliations with or involvement in any organization or entity with a direct financial interest in the subject matter or materials discussed in the manuscript.

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PAPER

FATTY ACID PROFILE OF POLISH MEAT PRODUCTS

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ABSTRACT

The aim of the study was to determine and compare the fatty acid composition of minced meat, smoked cooked hams and smoked dry pork sausages available on the Polish market. The fatty acid profile was determined using gas chromatography. Among all fatty acids found in the meat products the highest share had C18:1 n-9, C16:0, C18:0, C18:2 n-6, and C16:1. PUFA accounted for 8.7, 9.2 and 9% of total fatty acids whereas linolenic acid for 0.6, 0.7 and 0.3% in hams, sausages and minced meat, respectively. Minced meat had the highest n-6/n-3 ratio (22.5) compared to hams and sausages (15.5 and 13.9, respectively).

- Keywords: fatty acids, pork, minced meat, sausage, ham -

INTRODUCTION

Meat and meat products are an important source of high value animal proteins in the human diet. The most often-consumed meat (kg per person) in the world is pork (MURPHY *et al.* 2011). In Poland, pork is also the most popular, followed by poultry and beef. In 2011, the average consumption of pork per person was 42.5 kg as compared to 25.0 kg of poultry and 2.1 kg of beef (CENTRAL STATISTICAL OFFICE, 2012). Pork is sold as culinary meat, raw meat products (such as minced meat) and processed meat (such as cooked hams and sausages). In 2011, the monthly consumption of processed meat products (sausages and other preparations, produced from porcine meat) was 2.3 kg per person compared to fresh, chilled or frozen meat, including pork, poultry and beef (3.0 kg per person). The monthly consumption of high quality cured meat products and sausages (such as smoked cooked hams and smoked sausages) is continuously increasing (from 0.54 kg/person in 2005 to 0.65 kg/person in 2011) (CENTRAL STATISTICAL OFFICE, 2012).

Pork in the human diet is a source of protein, fat, vitamins (thiamin, riboflavin, niacin, vitamin B6, B12) and minerals (selenium, phosphorus, potassium, zinc) (MURPHY *et al.* 2011). Consumers whose diets contain fresh pork intake similar amounts of total fat, saturated fat and more protein, selenium, thiamin and vitamin B6 than consumers who did not eat fresh pork (MURPHY *et al.* 2011).

For over three decades, consumers have been encouraged to select lean or low-fat meat and meat products to lower the consumption of fat and cholesterol (MURPHY *et al.*, 2011). According to Regulation (EC) No 1924/2006 of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods, meat products that contain less than 3 g of fat can be described on a label as low fat. The term "low in saturated fat" can be used on the label of a product only if the sum of saturated fatty acids and *trans*-fatty acids does not exceed 1.5 g per 100 g of solid product and the sum of saturated fatty acids and *trans*-fatty acids provide no more than 10 % of energy.

The fat content in a porcine carcass depends on the breed, weight and diet of animals (WAJDA *et al.*, 2008; WOOD *et al.*, 2004). Pork, particularly processed porcine products, is considered unhealthy due to the high content of fat and cholesterol (REIG *et al.*, 2013). However, consumers can choose low fat cuts, such as tender loin, butt portions or low fat porcine products. Cooked ham is produced from pork leg, which is composed 68% of meat tissue, 5% of intermuscular fat, 19% of skin and subcutaneous fat and 8% of bones (WINIARSKI *et al.*, 2004). Fat content in ham meat tissue is only about 1.2-1.8 % (MIGDAŁ *et al.*, 2007). Intramuscular fat is often removed while preparing single muscles for cooked ham production and that is why cooked ham contains about 1.7% of fat (CHENG and SUN 2007). Sausages are produced from meat of different fat content and connective tissue, depending on sausage recipe (OLSZEWSKI, 2007) and that is why the fat content can even reach 35% (MAKAŁA *et al.*, 2007). According to the current nutrient recommendations, consumers should pay attention not only to the fat concentration in products but also to the fatty acid composition of fat and choose a diet low in saturated fat (LICHTENSTEIN, 2011). The fatty acid composition of the meat products depends on the fatty acid composition of meat and animal fat. As shown by GRZEŚKOWIAK *et al.* (2010), the fatty acids of pork loin were composed in 37% of saturated fatty acids (SFA), 62% of unsaturated fatty acids (UFA), 48% of monounsaturated fatty acids (MUFA) and 14% of polyunsaturated fatty acids (PUFA). SFA contribute to cardiovascular disease development caused by elevated blood pressure, increased LDL cholesterol and triacylglycerols content in serum (SERRANO *et al.* 2007), MUFA have a beneficial, or at least neutral, impact on the cardiovascular system, whereas PUFA are essential for the accurate functioning of the human body by stimulating the immunological system (WEBB and O'NEIL, 2008).

There are many reports concerning the fatty acid profile of porcine meat (MIGDAŁ *et al.*, 2007; GRZEŚKOWIAK *et al.*, 2010; BOSCH *et al.*, 2012) and the possibility to modify it by different additives used in animal feeding (WOOD *et al.* 2008; ROTOLO *et al.* 2011) and in meat products (TRINDADE, 2011; GRELA and KOWALCZUK, 2009; BAGGIO and BRAGAGNOLO, 2006; FERNÁNDEZ *et al.*, 2007; HADORN *et al.*, 2008). However, not many of them refer to smoked meat products that are very popular in Poland and are also exported to many European countries such as Ireland (http://dublin.trade.gov.pl), Great Britain and the USA (www.exporter.pl). Thus, there is still little information about the fatty acid composition of such products available on the Polish market. Therefore, the aim of the study was to determine and compare the fatty acid profile of three groups of porcine products: minced meat, smoked cooked hams and smoked dry sausages available on Polish market.

MATERIALS AND METHODS

Minced pork (n=15), smoked cooked hams (n=16) and smoked dry sausages (semi-coarse ground and coarse ground, n=11) were purchased from local supermarkets in amounts ranging from 500 to 750 g. All meat products analyzed in the present study were purchased in individual packages. Products within each group were produced by different manufactur-

ers or came from different production batches. Samples were delivered to the laboratory of the Chair of Commodities and Food Analysis and kept at 4°C±1 until analyses but no longer than 24 h. Before analyses, samples of minced meat were mixed to standardize its composition, whereas hams and sausages were ground through a 3-mm mesh. Samples were analyzed in duplicate.

The fat content in each product was determined according to PN-ISO 1444 (POLISH COMMITTEE FOR STANDARDIZATION 2000). Analyses were conducted in duplicate. To determine the fatty acid composition, fat was extracted from comminuted samples using a mixture of chloroform and methanol (2:1, v/v) according to the Folch method (FOLCH *et al.*, 1957). The methyl esters of fatty acids were prepared using a mixture of chloroform, methanol and sulfuric acid (PEISKER, 1964). Fat (50-60 mg) was placed into an ampule using a glass capillary tube and 1.5 mL of the methylating mixture (methanol-chloroform-concentrated sulfuric acid in the ratio 100:100:1 by volume) was added. The ampule was closed by melting the tip over a gas flame burner. The ampule was cooled at ambient temperature, checked for tightness and warmed in a boiling water bath for approx. 90 min. The content of the ampules were shaken every 15 min. The obtained esters were separated, identified and quantified using 7890A (Agilent Technologies Inc., Santa Clara, CA, USA) gas chromatograph with flame-ionization detector and Supelcowax 10 capillary column (30 m long, 0.32 mm of internal diameter and 0.25 μm film thickness, Supelco, Bellefonte, PA, USA). As a carrier gas, helium was used at a flow speed of 1.2 mL/min and a split ratio of 50:1. The detector and the injector were maintained at 250° and 225°C, respectively. The oven and column temperature was constant and equaled 195°C. The peaks were identified by comparing the retention times. As a reference material was used BCR No 163 (Blef/pig Fat blend).

Data analysis consisted of several steps. In the first step, the normal distribution of the results in the populations was tested using a Shapiro-Wilk test. When *p* values were higher than 0.05, the distribution was considered normal and the next step was to test the equality of group variances using a Brown-Forsyth test. If it resulted in *p* values higher than 0.05, the variances were considered as equal. In this case, to compare groups of means, an analysis of variance (F-test) was applied along with Duncan's test to distinguish significant differences. Duncan's test was conducted only if an F-test resulted in *p* values lower than 0.05. If the distribution of the data was different from normal or the groups of variances were not equal, a non-parametric Kruskal-Wallis test was used. All analyses were conducted in Statistica 8.0 (StatSoft Inc.).

RESULTS AND DISCUSSION

Minced meat, hams and sausages contained *ca.* 25.7% (± 3.2), 3.8% (± 1.3) and 21.7% (± 2.4) of fat, respectively. The fat content in all tested samples of minced meat, hams and sausages was in agreement with the Polish and European Union regulations. According to the Polish Standard PN-A-82007 (POLISH COMMITTEE FOR STANDARDIZATION, 1996), the fat content in dry sausages should not exceed 30% for coarse ground dry sausages and 45% for semi-coarse ground sausages. For hams, the maximum fat content is 10% (REGULATION OF THE MINISTER OF AGRICULTURE AND RURAL DEVELOPMENT of 24th May 2004). The fat percentage in minced meat containing pork should be under 30% (COMMISSION REGULATION (EC) No 1162/2009).

Fatty acids composition of minced pork, smoked cooked ham and dry sausage was presented in Table 1. Figs. 1A-1C show representative chromatograms for samples of minced meat, ham and sausages. The main fatty acids found in the products were C18:1(n-9), C16:0, C18:2 (n-6), C18:0 and C16:1. The same was noted by BAGGIO and BRAGAGNOLO (2006) who investigated fatty acid profile of such commercial products as beef jerky, Italian-type salami, chicken and Chester mortadellas.

In the Polish commercial products, the predominant SFA were palmitic (C16:0, approx. 26%) and stearic (C18:0, approx. 12%) acids (Table 1). In traditional Spanish meat products such as chorizo, longaniza, lomo sajonia, cinta de lomo and morcilla, SFA ranged from 36 to 43%, palmitic acid (C 16:0) accounted for 23-24% and stearic acid (C18:0) for 12-15% (JIMÉNEZ-COLMENERO *et al.*, 2010). SFA differ in terms of their impact on human health. Stearic acid does not exhibit an artherogenic effect, whereas palmitic and myristic acids do (WILLIAMSON *et al.*, 2005). In traditional Spanish meat products, palmitic and myristic acids accounted for 25% of the total fatty acids (JIMÉNEZ-COLMENERO *et al.*, 2010). The same was noted for the Polish meat products. Moreover, there were no differences between minced meat, hams and sausages in terms of palmitic and myristic acids (Table 1).

Significant differences between minced meat, sausages and hams were noted in one saturated fatty acid (C18:0), one monounsaturated fatty acid (C18:1) and in four polyunsaturated fatty acids (C18:2, C18:3, C20:2, C20:4). This accounted for the differences in SFA, UFA, MUFA and PUFA between the products (Table 1). The hams had a lower share of SFA (38.96%) and higher UFA (61.04%) compared to the sausages (41.35 and 58.65%, respectively) and the minced meat (40.80 and 59.20, respectively). The samples of minced meat and hams had higher MUFA (52.47 and 52.33%, respective-

Table 1 - Fatty acid compositions (% of total identified) of minced pork (n = 15), smoked cooked ham (n = 16) and smoked dry sausage (n = 11).

Fatty acid	Minced pork	Smoked cooked ham	Smoked dry sausage
C14:0 myristic acid	1.46±0.09 a	1.46±0.14 a	1.51±0.18 a
C15:0 pentadecanoic acid	0.09±0.07 a	0.05±0.02 a	0.07±0.02 a
C16:0 palmitic acid	25.90±1.03 a	25.59±1.27 a	25.99±0.29 a
C17:0 margaric acid	0.31±0.12 a	0.28±0.06 a	0.32±0.06 a
C18:0 stearic acid	12.87±1.29 ab	11.42±2.87 b	13.30±0.50 a
C20:0 arachidic acid	0.16±0.03 a	0.16±0.02 a	0.17±0.02 a
C16:1 palmitoleic acid	3.15±0.42 a	3.45±0.59 a	3.03±0.23 a
C17:1 margaric-oleic acid	0.33±0.11 a	0.34±0.07 a	0.33±0.05 a
C18:1 oleic acid (n-9)	48.22±2.63 a	47.78±2.74 a	45.37±1.12 b
C20:1 eicosenic acid (n-9)	0.76±0.14 a	0.77±0.10 a	0.73±0.07 a
C18:2 linoleic acid (n-6)	5.68±1.57 b	7.28±1.26 a	7.89±0.83 a
C18:3 linolenic acid (n-3)	0.32±0.13 b	0.56±0.20 a	0.70±0.41 a
C20:2 eicosadienoic acid (n-6)	0.23±0.05 b	0.26±0.05 ab	0.29±0.03 a
C20:4 arachidonic acid (n-6)	0.51±0.14 a	0.60±0.31 a	0.31±0.04 b
SFA	40.80±2.07 a	38.96±2.59 b	41.35±0.77 a
UFA	59.20±2.07 b	61.04±2.59 a	58.65±0.77 b
MUFA	52.47±2.81 a	52.33±2.85 a	49.45 ±1.24 b
PUFA	6.73±1.75 b	8.70±1.54 a	9.20±1.07 a
Σ n-6	6.42±1.65 b	8.14±1.40 a	8.49±0.86 a
Σ n-3 (C18:3 only)	0.32±0.13 b	0.56±0.20 a	0.70±0.41 a
n-6/n-3	22.53±7.61 a	15.52±3.55 b	13.87±3.60 b

ab - means in rows with different letters differ at $p < 0.05$.
SFA, saturated fatty acids (sum of C14:0, C15:0, C16:0, C17:0, C18:0); UFA, unsaturated fatty acids (sum of C16:1, C17:1, C18:1, C20:1, C18:2, C18:3, C20:2, C20:4), MUFA, monounsaturated fatty acids (sum of 16:1, C17:1, C18:1, C20:1); PUFA, polyunsaturated fatty acids (sum of C18:2, C18:3, C20:2, C20:4); n–6/n–3 ratio, (C18:2+C20:2+C20:4)/C18:3.

ly) than sausages (49.45%). The minced meat had lower PUFA, Σ n-6 and Σ n-3 compared to the hams and sausages. Different results were obtained by HADORN *et al.* (2008), who noted that pork hamburgers had a higher amount of PUFA (12.6%) compared to Vienna Sausage (9.3%), salami (7.6%) and raw-cured bacon (8.1%). Polish minced meat, smoked cooked hams and smoked sausages had higher MUFA (from 49.5 to 52.5%) amounts compared to the

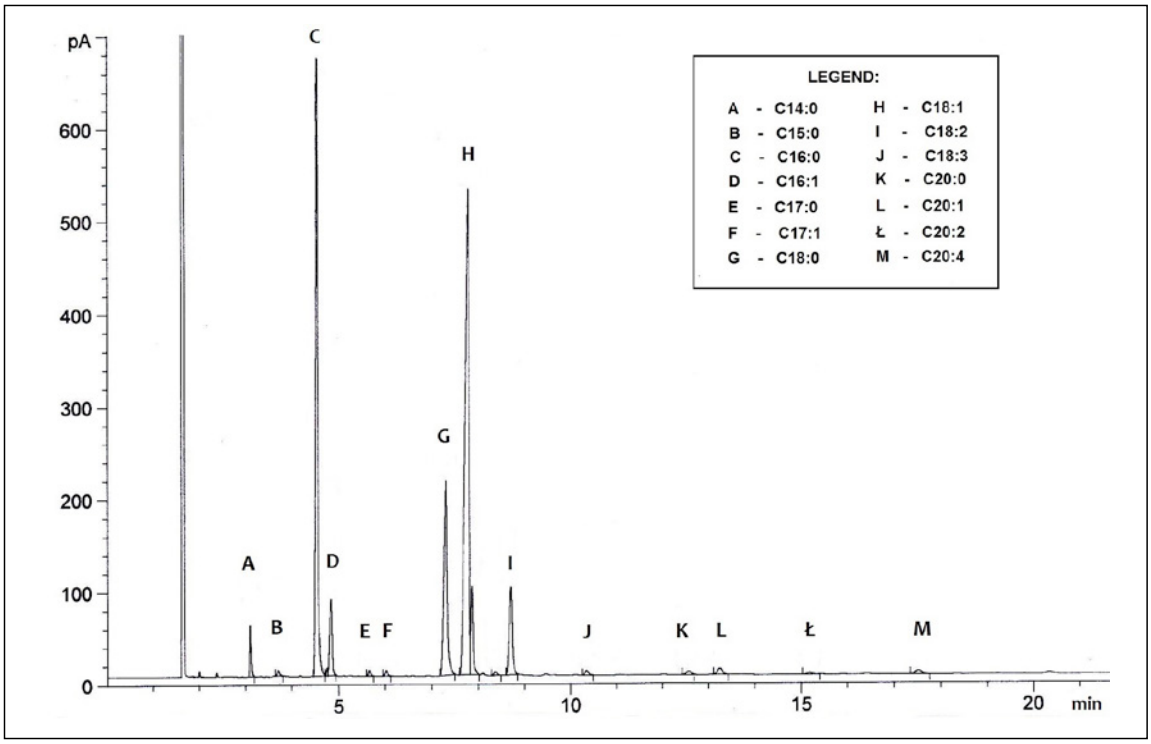


Fig. 1A - Chromatogram of a minced pork meat sample.

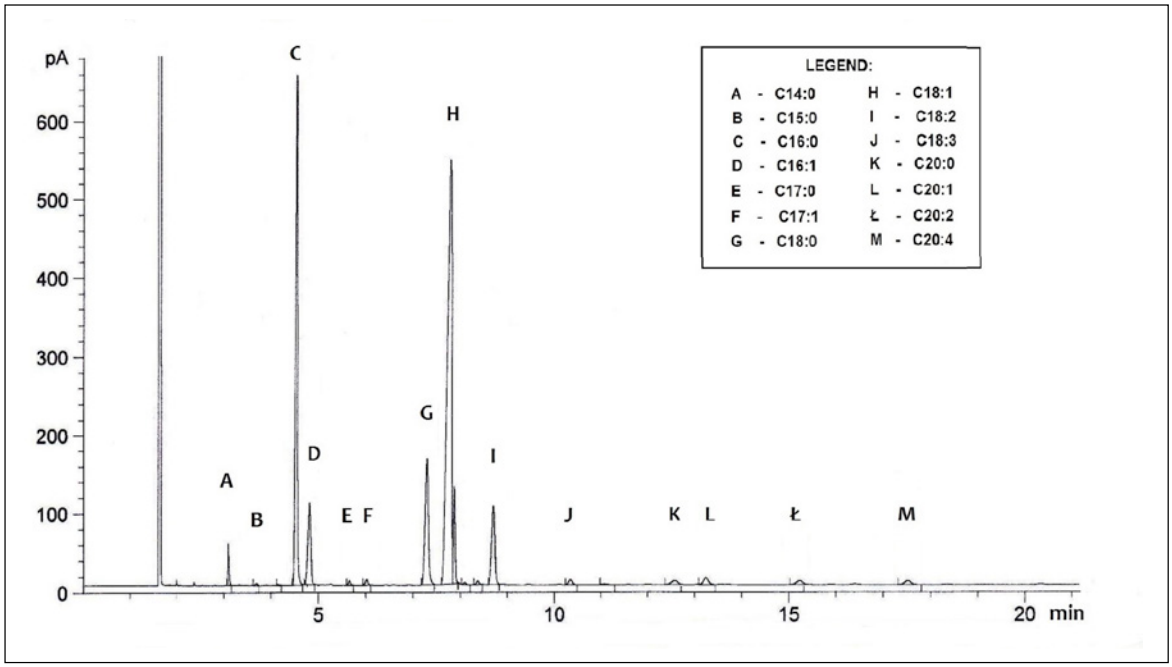


Fig. 1B - Chromatogram of a smoked cooked ham sample.

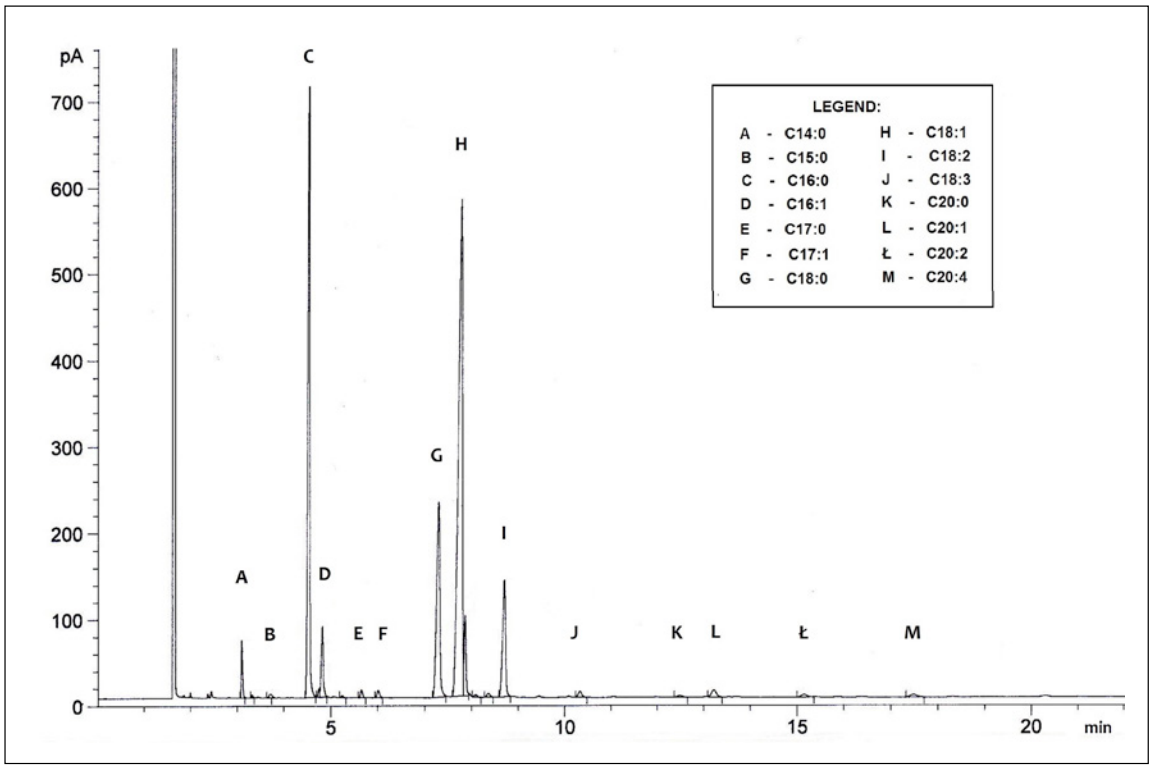


Fig. 1C - Chromatogram of a smoked dry sausage sample.

Spanish sausages (from 40 to 48%), but in both Polish and Spanish products oleic acid was predominant (45-48 and 39-44%, respectively). Polish and Spanish meat products also differed in PUFA, which in Polish products ranged from 7 to 9% and in Spanish from 13 to 20% (JIMÉNEZ-COLMENERO *et al.* 2010). The fatty acid composition of meat can be improved for humans by feeding pigs diets with an addition of preparations rich in unsaturated fatty acids, such as linseed oil (GRZEŚKOWIAK *et al.*, 2008; GUILLEVIC *et al.*, 2009), pure docosahexaenoic acid (DHA) from marine source oil (SÁRRAGA *et al.*, 2007) or dietary conjugated linoleic acid (CLA) (LO FIEGO *et al.*, 2005).

The recommended n-6/n-3 ratio is less than 4 (JIMÉNEZ-COLMENERO, 2007). A higher n-6/n-3 ratio is not beneficial for human health because

of the possibility to promote pathogenesis of cardiovascular disease, cancer, inflammatory and autoimmune diseases (JIMÉNEZ-COLMENERO, 2007). Sausages and hams had significantly a lower n-6/n-3 ratio (13.87 and 15.52, respectively) compared to the minced meat (22.53) (Table 1). In the Spanish traditional products, the n-6/n-3 ratio was also higher than recommended and ranged from 10 to 19 (JIMÉNEZ-COLMENERO *et al.*, 2010). As has been shown in many papers (ANSORENA and ASTIASARÁN, 2004; PELSER *et al.*, 2007), the n-6/n-3 ratio in meat products can be lowered by adding plant or fish oil during the production process.

CONCLUSIONS

There is currently great interest in fat content and the fatty acid profile of food products because of their health impact. Minced pork meat, smoked cooked hams and smoked dry sausages available on the Polish market, investigated in the present study, differed in terms of fat content. Fat content in all tested products was in agreement with the Polish and European Union (EU) regulations. According to the Regulation (EU) No 1169/2011 fat content should be indicated on the label of the food product along with the amount of saturated, monounsaturated and polyunsaturated fatty acids. The differences in fatty acid profile between the meat products tested in the present study were noted. The n-6/n-3 ratio in smoked cooked hams and smoked sausages was significantly lower than in minced meat. Hams and sausages had a higher share of PUFA and linolenic acid (C18:3) compared to the minced meat. The smoked hams had a lower share of SFA and higher UFA (including MUFA) than the sausages. However, no differences were found in the most important for human health fatty acids (PUFA) and the n-6/n-3 ratio between hams and sausages.

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PAPER

THE EFFECT OF EDIBLE COATING ON THE QUALITY OF SMOKED FISH

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ABSTRACT

The combined effect of protein based edible coating and vacuum packaging on the shelf-life of hot-smoked rainbow trout stored under refrigeration was investigated for a period up to 6 weeks. Edible coatings were produced from the protein sources (soy protein isolates (SPI), concentrate whey protein isolates (WPI), egg white powder protein (EP), wheat gluten (WG), corn protein (Z), gelatin (G), collagen (Co)) and from proteins of two different fish species (rainbow trout (RT) and Atlantic mackerel (AM)). Quality attributes monitored microbiological, sensory, chemical, color and texture parameters. Based on both microbiological and sensory analyses, treatments Co produced a shelf-life extension of 2-3 weeks.

- Keywords: edible coating, protein, shelf-life, smoked fish and quality -

1. INTRODUCTION

Rainbow trout is one of the most economically important freshwater-cultured fish species. It is popular in Turkey and the world due to its fast growth rate, palatability, and the nutritional quality of its flesh. Rainbow trout is the most cultured freshwater fish species in Turkey, and its production is 100239 tons/year. Its fresh and smoked consumption is quite common in Turkey, and it is also exported to European Countries (TUIK, 2012).

Smoking is a traditional method of preserving fish, since it delays microbiological and oxidative changes. The ability of the smoking process to preserve fish is due to the synergistic action of salt incorporation, the preservative effect of smoke compounds and dehydration during the smoking process (BANNERMAN, 1980).

Smoked fish are usually more perishable than other processed seafoods (dried, salted and marinated fish), and a considerable number of smoked fish are spoiled due to lack of good preservation (BREMMER, 2002). Factors affecting the quality of smoked fish can be explained as follows: microbial load of raw material, lipid value of raw material, type of smoking, salt concentration, smoking time and temperature, sawdust type, packaging type and storage temperature (ARVANITOYANNIS and KOTSANOPOULOS, 2012).

Besides traditional methods used to extend the shelf-life of hot smoked fish products, such as rapid chilling and cold storage, various strategies involving modified atmosphere packaging, high pressure and the use of organic acids or other antimicrobial agents have been proposed to inhibit spoilage and pathogenic microorganisms (ERKAN *et al.*, 2009; ERKAN *et al.*, 2011a, 2011b).

Consumer demand for safe, minimally processed, additive free, shelf-stable foods that retain the appearance, natural flavor, texture, and nutritional qualities of raw or fresh foods has been a driving force for edible coatings in new researches (DEBEAUFORT *et al.*, 1998).

The advantage of the application of edible coating includes inactivation of food spoilage and pathogenic microorganisms at low and ambient temperatures with minimal effects on the flavor and nutritional attributes of the product (DUTTA *et al.*, 2009). The exact mechanism of the antimicrobial activity of edible coatings can be explained as follows: (1) the activation of several defense processes in the host tissue by the coating material, acting as a water-binding agent and inhibiting various enzymes by blocking their active centers; (2) the action of coating material as a chelating agent, selectively binding metals and then inhibiting the production of toxins and microbial growth; (3) the formation, generally by high molecular weight coating material, of an impervious polymeric layer on the surface of the cell, thereby altering cell permeability and blocking the entry of nutrients into the cell; (4) the penetration of mainly low-molecular weight coating material into the cytosol of the microorganism to bind DNA, resulting in interference with the synthesis of mRNA and proteins; and (5) the adsorption and flocculation of electronegative substances in the cell by the coating material, disturbing the physiological activities of the microorganisms, causing their death. However, it is very important to mention that coating material is soluble only in acidic or alkaline media and therefore the effect of pH on microorganisms must be considered together with the effect of the coating material. Thus, the synergistic effect of the coating material/pH together is probably the most evident explanation of the antimicrobial effect of the coating material (DUTTA *et al.*, 2009; AIDER, 2010).

The most commonly used materials for edible film production are biopolymers such as carbohydrates (alginate, chitosan, carrageenan, etc.) and proteins (THARANATHAN, 2003). Even though several researchers have studied alginate and chitosan as an edible coating material for fishery products to enhance the quality (DUAN *et al.*, 2010; SONG *et al.*, 2011; MOHAN *et al.*, 2012), little information exists on the effect of protein-based coatings (ERKAN *et al.*, 2013). However, there is no study in the literature examining the effects of protein-based edible coating on the shelf-life of hot-smoked fillets during storage. From this regard, the aim of this study is to investigate the effects of protein-based edible coating obtained from the different natural protein sources for chemical, microbiological and sensorial properties of hot smoked rainbow trout fillets during cold storage.

2. MATERIALS AND METHODS

2.1. Raw material

Hot smoked fish were prepared from rainbow trout (*Oncorhynchus mykiss*). Rainbow trouts (268.27±24.01 g and 28.07±1.21 cm) were obtained from a freshwater fish farm of the Liman Company in Bozuyuk (Bilecik, Turkey). The fresh fish samples were packed in polystyrene boxes with crushed ice, and then transferred to the laboratory. Fish were harvested, beheaded and gutted manually, and then washed. The samples (140 gutted fish) were immersed in brine at a ratio of 1:1 (w/w) for 18 h at 2°C. The brine contained 6.5% NaCl (sodium chloride). After brining, the samples were submerged in chilled tap water for 1 h.

2.2. Smoking process

Smoke was produced by the combustion of oak sawdust. The processing time in the kiln was divided into four stages: (1) a preliminary drying of 15 min at room temperature (20°C); (2)

cooking period of 60 min at 75°C; (3) smoking and partial cooking period 120 min at 80°C; (4) after cooling for 60 min at room temperature.

2.3. Treatment, packaging and storage

2.3.1. Edible film coating preparations

2.3.1.1. Edible coatings from soy protein isolate

Soy protein isolate (SPI) has a reported minimum 90% protein content on a dry basis (Smart Kimya Company, Izmir, Turkey). SPI films were prepared according to the method advocated by DENAVI *et al.* (2009). Ten grams of the SPI were dissolved in a constantly stirred mixture of distilled water (200 mL) and glycerine (Merck, Darmstadt, Germany) (5 mL) for 15-20 min at 55°-60°C. The solution's pH was adjusted to 10.5±0.1 with 2 M NaOH (sodium hydroxide) solution. The film solutions were heated for 10-15 min at 75°-80°C in a constant-temperature water bath to denature the soy protein.

2.3.1.2. Edible coatings from whey protein isolate

Whey protein isolate (WPI) has a reported minimum 80% protein content on a dry basis (Smart Kimya Company, Izmir, Turkey). WPI films were prepared according to the method advocated by SARIKUS (2006). Ten grams of WPI were dissolved in a constantly stirred mixture of distilled water (200 mL) and glycerine (10 ml) for 15-20 min at 55°-60°C. The solution's pH was adjusted to 8.0±0.1 with 2 M NaOH solution (Merck, Darmstadt, Germany). The film solutions were heated for 15-20 min at 75°-80°C in a constant-temperature water bath.

2.3.1.3. Edible coatings from egg white powder protein

Egg white powder protein (EP) has a reported minimum 80% protein content on a dry basis (Smart Kimya Company, Izmir, Turkey). EP films were prepared according to the method advocated by GENNADIOS *et al.* (1997). Eighteen grams of the EP were dissolved in a constantly stirred mixture of distilled water (200 ml) and glycerine (9 ml) for 5 min at room temperature. The solution's pH was adjusted to 11.25 ±0.1 with 1 M NaOH solution. The film solutions were heated for 20 min at 45°C in a constant-temperature water bath.

2.3.1.4. Edible coatings from wheat gluten

Wheat gluten (WG) has a reported minimum 75-82% protein content on a dry basis (Smart Kimya Company, Izmir, Turkey). WG films were prepared according to the method advocated by TANADU-PALMU (2000). Fifteen grams of the WG were dissolved in a constantly stirred mixture of distilled water (90 mL), ethanol (Merck, Darmstadt, Germany) (110 mL) and glycerine (7.5 mL) for 15 min at 55°C. The solution's pH was adjusted to 4.0±0.1 with a 50% glacial acetic acid solution. The film solutions were heated for 15 min at 70°C in a constant-temperature water bath.

2.3.1.5. Edible coatings from corn protein (zein)

Zein (Z) (minimum 90% protein content on a dry basis) was obtained from Sigma Aldrich (USA) (Smart Kimya Company, Izmir, Turkey). Z films were prepared according to the method advocated by BAYSAL *et al.* (2009). Seven and a half grams of Z was dissolved in a constantly stirred mixture of ethanol (180 ml) and glycerine (3 ml) for 30 min at 75°-80°C.

2.3.1.6. Edible coatings from gelatin

Gelatin (G) has a reported minimum 83% protein content on a dry basis (Smart Kimya Company, Izmir, Turkey). G films were prepared according to the method advocated by THOMAZINE *et al.* (2005). Four grams of G were dissolved in a constantly stirred mixture of distilled water (200 mL) and glycerine (2.2 mL) for 30 min at 55°-60°C.

2.3.1.7. Edible coatings from collagen

Collagen (Co) has a reported minimum 90% protein content on a dry basis (Smart Kimya Company, Izmir, Turkey). Co films were prepared according to the method advocated by HO *et al.* (2001). Three grams of Co were dissolved in a constantly stirred mixture of 3% glacial acetic acid (Merck, Darmstadt, Germany) (200 mL) and glycerine (1.5 mL) for 30 min at 75-80°C.

2.3.1.8. Edible coatings from rainbow trout

Rainbow trouts (RT) (*Onchorcynhus myksis*) with an average weight of 150-200 g were obtained from the Istanbul Fish Market. The fish were stored in ice and offloaded approximately 12 h after capture. The fish were transported in ice to the Department of Seafood Processing and Quality Control, Faculty of Fisheries of Istanbul University within 2 hours. Upon arrival, whole fish were immediately washed and stored in ice with a fish/ice ratio of 1:2 (w/w). The mixture of ice and fish were placed in polystyrene boxes, which were kept at room temperature (30°-32°C). Fish samples were filleted manually. Thirty grams of fish, distilled water (181.32 mL) and glycerine (2.77 mL) mixed using a blender, were filtered. This solution was stirred for 10-15 min at 75°-80°C. The solution's pH was adjusted to 3.0±0.1 with a 50% glacial acetic acid solution. The film solutions were heated for 15 min at 75°-80°C in a constant-temperature water bath (CUQ *et al.*, 1997). In this study, the minimum protein content of the RT was determined as 18.48%.

2.3.1.9. Edible coatings from Atlantic mackerel

Frozen Atlantic mackerels (AM) (*Scomber scombrus*) with an average weight of 150-200 g was obtained from the Istanbul Fish Market.

Fish were stored in ice and offloaded approximately 12 h after capture. The fish were transported in ice to the Department of Seafood Processing and Quality Control, Faculty of Fisheries of Istanbul University within 2 hours. Thawed fish samples were filleted manually. Thirty grams of fish, distilled water (200 ml) and glycerine (2.0 mL) were mixed using a blender and then filtered. This solution was stirred for 10-15 min at 75°-80°C. The solution pH was adjusted to 3.0±0.1 with a 50% glacial acetic acid solution. The film solutions were heated for 15 min at 75°-80°C in a constant-temperature water bath (CUQ *et al.*, 1997). In this study, the minimum protein content of the AM was determined as 18.50%.

2.3.2. Packaging and storage

Smoked fish fillets were randomly separated into ten groups. The first group was untreated and used as the control (C). The fillets of the each group were treated with edible SPI, WPI, EP, WG, Z, G, Co, RT, AM solutions. Solutions were applied to the surface (both sides) of each fillet using a silicone brush for each group. After treatment, fillets were allowed to dry for 40 min on a sterile stainless steel mesh screen at ambient temperature (20°C).

Three treated fillets for each group were packaged in Low Density Polyethylene/Polyamide (LDPE/PA) barrier pouches. The plastic film pouch was obtained from the Polinas Plastic Company (Manisa, Turkey). The characteristics of the plastic film bags were as follows: thickness: 90 μm, total light transmission: 30%; O_2 transmission: 160 cm^3/m^2/day/atm at 75% RH (relative humidity), 25°C; vapor permeability: 8.50 g/m^2/day at 100% RH, 25°C. The pouches were heat sealed using a vacuum-sealing machine (Henkovac vacuum packaging machine, ML Hertogenbosch, Netherlands). Pouches were kept refrigerated (2°C) for a period of 6 weeks.

2.4. Microbiological analysis

2.4.1. Sample preparation

Smoked fish (25 g) obtained from each smoked fish fillet, were transferred aseptically to a Stomacher bag (Seward Medical, London, UK) containing 225 mL of 0.1% peptone water (Merck, 107228) and homogenized for 60 s using a Lab Blender 400, Stomacher at high speed (Stomacher, IUL Instrument, Spain).

2.4.2. Microbiological media and count

To obtain the microbial counts, 0.1 mL samples of serial dilutions (1:10, diluents, 0.1% peptone water (Merck, 107228, Darmstadt, Germany) of fish homogenates were spread on the surface of agar plates. TVC was determined using plate count agar (PCA, Merck, 105463) after incubation for 24-48 h at 37°C. PCA was used for psychrotrophic bacteria and incubated at 7°C for 10 days. TAC was determined by PCA incubated under anaerobic conditions (with a 5% CO_2 incubator, HF 90 model, Shanghai, China) at 30°C for 24-48 h. Yeasts and molds were enumerated using Dichloran Rose Bengal Chloramphenical (DRBC, Merck 10046) after incubation at 25°C for 3 days in the dark. Results are expressed as a logarithm of colony forming units (log cfu) per gram of sample (BELL *et al.*, 2005). Thus, the detection limit of total mesophilic, psychrophilic and anaerobic bacterial counts were <1.00 log cfu/g. All the analyses were performed in duplicate. For anaerobic sulphite-reducing *Clostridium* count, 25 g of sample were homogenized and incubated at 30°C for 14 days in Differential Reinforced Clostridial Broth (DRCM, Merck 1.11699) under anaerobic conditions. Results were expressed as log MPN/g of samples. Tryptose sulphite cycloserine agar (TSC Agar; Merck 111972) supplemented with D-cycloserine (Merck, 100888) was used for the enumeration of *Clostridium perfringens*, and the plates were incubated at 30°C for 24-48 h under anaerobic conditions. *Clostridium botulinum* was enumerated on Anaerobic Agar acc. to Brewer (Merck, 105452 after preenrichment in Cooked Meat Medium (R. C. Medium, Himedia M 149, Mumbai, India) at 35°C for 5-10 days following incubation at 35°C for 45-48 h (ICMSF, 1986).

2.5. Sensory analysis

The attributes of hot-smoked fish were evaluated by a panel of five experienced judges on each week of sampling. Panelists were laboratory trained. Sensory evaluation was conducted in individual booths under controlled conditions of light, temperature and humidity. Sensory analysis was performed using the methods of MOHAN *et al.* (2012). Smoked fish were assessed on the basis of appearance, odor, taste and texture characteristics using a nine point descriptive scale. A score of 9-7 indicated "very good quality", a score of 6.9–5.0 "good or acceptable quality", a score of 4.9–1.0 "bad or unacceptable quality". The skins of smoked fish were removed for the physical and chemical analysis, and were homogenized using a food processor.

2.6. Chemical analysis

TVB-N (mg/100g fish flesh) was determined according to the method described by ANTONACOPOULOS and VYNCKE (1989). TBA-i values (mg malondialdehyde (MDA)/kg) were measured using the method of ERKAN and OZDEN (2008).

2.7. Colour and texture profile

Three fillets for each group were divided for colour and texture analysis. The colour and texture were measured twice (the first measurement was taken at the beginning of storage after the smoking process, and the second measurement was taken in response to the result of the weekly sensory analysis that were considered unacceptable). The colour of the smoked fish samples was determined with the help of a Konica Minolta Chromo Meter (model CR 400/410; Minolta, Osaka, Japan) from the flesh of the anterio-dorsal region of each fillet after the smoking process. L^* (brightness), a^* (+ a, red; – a, green) and b^* (+ b, yellow; – b, blue) values were measured. The colorimeter was calibrated using white references (CR-A44; L^*: 50.50, a^*: 0.37, b^*: -1.26). ΔE^* were calculated according to the following formulas with measured L^*, a^* and b^* values. $\Delta E^* = (\Delta L^2+\Delta a^2+\Delta b^2)$ (GERDES and SANTOS VALDEZ, 1991).

Texture analysis was undertaken using 3×3 cm^2 samples from each group, taken from the flesh of the Ontario-dorsal region of each fillet. Textural analyses of the fillets were performed by CT3-1500 Texture Analyser (Brookfield Texture Analyser, Guangzhou, China) equipped with a load cell of 1.5 kg. Condition of apparatus: Test type: Texture profile analysis (TPA) with compression, Test target: Load, Target value: 2.000 N, Hold time: 5 s, Trigger load: 0.020 N, Test speed: 1.00 mm/second, and Probe type: TA 50. Measured parameters were values of fracturability (N), hardness (N), springiness (mm), adhesiveness (N×mm), gumminess (N), chewiness (N×mm), cohesiveness and resilience. Three measurements were taken from three fillets belong to each group.

2.8. Statistical analysis

The resulting analysis data were evaluated by using an IBM SPSS Statistics 20® program. The results were given as an average ± standard deviation. The one-way analysis of variance (One-Way ANOVA) was applied. In the data parametric assumptions for multiple comparisons occurred. The Tukey test was used to locate the sources of the differences found within different groups by this test. $P<0.05$ variation was accepted as the significant discrepancy between the groups and the parameters depending on storage.

3. RESULTS

Fresh and low microbial-load raw materials are preferred for high-quality smoked fish products. The high temperature during smoking reduces the number of microorganisms in smoked fish. However, some mesophilic, psychrotrophic microorganisms or spores can survive in hot smoked fish products. The microbiological quality of smoked fish is related to the initial bacterial contamination of the raw material, the salt concentrations in the flesh of the smoked fish, pre-drying and smoking time (ARVANITOYANNIS and KOTSANOPOULAS, 2012). Lactic acid bacteria and fungi are important organisms that grow rapidly during preservation/storage. Smoked fish may be contaminated with microorganisms that affect the quality and safety of the product during packaging and storage. Smoked fish products are usually sold in vacuum packing and anaerobic bacteria *Clostridium* spp. should be considered in terms of these products' quality assurance (TRUELSTRUP HANSEN *et al.*, 1995).

Microbiological parameters of hot smoked rainbow trout fillets coated with edible protein film are presented in Table 1. The tolerated microbiological value of TVC is usually accepted as 5.70 log cfu/g for fish and fish products (ICMSF, 1986). TVC of fish products is typically 7–8 log cfu/g at the point of sensory rejection. However, most consumer safety standards report much lower TVC (like 6 log cfu/g) for indices of acceptability. This value is considered as the microbial criterion in determining of shelf-life of foods (OLAFSDOTTIR *et al.*, 1997). Similar observations have been published. The recommended aerobic plate count limit for fresh fish consumption ranges between 5–7 log cfu/g (MONTAGNER *et al.*, 2005). It is reported that $3x10^6$ cfu/g (6.48 log cfu/g) is usually the microbial safety criterion limit for the mesophilic aerobic bacteria load of fish (CHANG *et al.*, 1998). This limit was exceeded in WPI, C-EP, and G group samples at the 2nd, 3rd and 4th weeks, respectively. None of the SPI, WG, Z, Co, RP and AB group samples exceeded this level during the storage period. This antimicrobial effect may be due to acetic acid (in the WG, Z, Co, RP and AB groups) and ethyl alcohol (in the Z group) added to film solutions during preparation. Thus may be due to the fact that acetic acid increased acidity (reducing the pH to 3-4) and ethyl alcohol had an antimicrobial effect. The cause the lack of mesophilic aerobic bacteria growth in the SPI group may be due to antimicrobial isoflavones (especially genistein) contained in soybeans (Dixon and Ferreira, 2002; Hong *et al.* 2006).

KOLSARICI and OZKAYA (1998) reported that the initial total mesophilic aerobic bacteria count of hot smoked rainbow trout samples was 4.32 log cfu/g, and this value increased to 7.36 log cfu/g after 48 days of refrigerated storage. CAKLI *et al.* (2006) reported the initial total mesophilic aerobic bacteria count of 2.6 log cfu/g, and this value increased to 7.6 log cfu/g after 40 days refrigerated storage. BILGIN *et al.* (2008) reported that the unacceptable total mesophilic aerobic bacteria count of hot smoked gilt-head

bream samples was 6.55 log cfu/g on the 60th day of storage. ERKAN (2012) reported the initial mesophilic aerobic bacteria count of hot smoked rainbow trout of 3 log cfu/g and this value increased to 6.65 log cfu/g after 7 weeks of storage in a refrigerator (2°C).

The sensory attributes of quality and the shelf life of smoked fish are mainly affected by the initial microbial contamination, processing conditions (cold and hot smoking), handling of the product after processing, packaging type and storage temperature (KOLSARICI and OZKAYA, 1998). The microflora of hot smoked fish is dominated by psychotropic Gram-negative rod-shaped bacteria belonging to the general *Pseudomonas* and *Aeromonas*. These psychotropic bacteria are the specific spoilage bacteria of hot smoked fish products (KOLSARICI and OZKAYA, 1998; BELL *et al.*, 2005). The spoilage value of psychrotrophic bacteria count was reported as 7 log cfu/g for fish fillets (NOSEDA *et al.*, 2012).

The psychrotrophic bacteria count of WPI, C-EP and G group samples exceeded the limit on the 2nd, 3rd and 4th week of storage (7.69 log cfu/g, 7.51-7.52 log cfu/g and 6.78 log cfu/g) (Table 1). In SPI and WG, Z, Co, RT, AM group samples on the 8th week of storage and WG, Z, Co, RT and AM group was found to be 3.09 log cfu/g and <1 log cfu/g, respectively. KOLSARICI and OZKAYA (1998) found that the count of psychrotrophic bacteria was 6.25 log cfu/g in hot smoked trout stored in a vacuum pack for 48 days at 4°C, whereas BILGIN *et al.* (2008) discovered that the psychrotrophic bacteria count was 6.36 log cfu/g in hot smoked trout stored in a refrigerator for 60 days.

TAC showed a similar pattern in mesophilic

Table 1 - Microbiological parameters of hot smoked rainbow trout fillets coated with protein based film during storage at 2°±2°C.

	Storage time (week)	C	SPI	WPI	EP	WG
Total mesophilic bacteria count (log cfu/g)	**0**	3.63±0.02^{Aa}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}
	1	5.12±0.01^{Ab}	<1.00±0.00^{Ba}	5.01±0.13^{Ab}	3.99±0.07^{Cb}	<1.00±0.00^{Ba}
	2	6.03±0.02^{Ac}	<1.00±0.00^{Ba}	**7.46±0.03^{Cc}**	5.41±0.02^{Dc}	<1.00±0.00^{Ba}
	3	**7.13±0.07^{Ad}**	2.83±0.02^{Bb}	8.31±0.04^{Cd}	**6.94±0.05^{Dd}**	<1.00±0.00^{Ea}
	4	7.50±0.33^{Ae}	<1.00±0.00^{Ba}	8.55±0.04^{Ce}	7.53±0.07^{Ae}	<1.00±0.00^{Ba}
	5	7.96±0.04^{Af}	<1.00±0.00^{Ba}	8.60±0.17^{Cef}	7.65±0.05^{Df}	<1.00±0.00^{Ba}
	6	6.60±0.00^{Ag}	2.80±0.06^{Bb}	8.77±0.01^{Cf}	8.75±0.02^{Cg}	<1.00±0.00^{Da}
Total psychrotrophic bacteria count (log cfu/g)	**0**	3.14±0.01^{Aa}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}
	1	5.39±0.01^{Ab}	<1.00±0.00^{Ba}	5.67±0.07^{Cb}	3.87±0.08^{Db}	<1.00±0.00^{Ba}
	2	5.46±0.00^{Ab}	<1.00±0.00^{Ba}	**7.69±0.01^{Cc}**	5.38±0.02^{Dc}	<1.00±0.00^{Ba}
	3	**7.51±0.00^{Ac}**	2.73±0.01^{Bb}	8.35±0.02^{Cd}	**7.52±0.00^{Ad}**	<1.00±0.00^{Da}
	4	7.88±0.03^{Ad}	<2.00±0.00^{Bc}	8.61±0.02^{Ce}	7.30±0.00^{De}	<1.00±0.00^{Ea}
	5	8.34±0.07^{Ae}	3.16±0.02^{Bd}	8.81±0.12^{Cfg}	7.47±0.00^{Dd}	<1.00±0.00^{E}
	6	6.81±0.07^{Af}	2.75±0.07^{Bb}	8.85±0.00^{Cg}	8.68±0.12^{Df}	<1.00±0.00^{Ea}
Total anaerobic bacteria count (log cfu/g)	**0**	3.29±0.01^{Aa}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}
	1	5.40±0.02^{Ab}	<1.00±0.00^{Ba}	5.64±0.12^{Cb}	4.50±0.10^{Db}	<1.00±0.00^{Ba}
	2	5.40±0.08^{Ab}	<1.00±0.00^{Ba}	**7.62±0.02^{Cc}**	5.50±0.00^{Dc}	<1.00±0.00^{Ba}
	3	6.40±0.01^{Ac}	2.78±0.01^{Bc}	8.41±0.04^{Cd}	**7.39±0.02^{Dd}**	<2.00±0.00^{Eb}
	4	**6.81±0.04^{Ad}**	<2.00±0.00^{Bd}	8.29±0.11^{Cd}	7.34±0.11^{Dd}	<2.00±0.00^{Bb}
	5	8.14±0.01^{Ae}	<2.00±0.00^{Bd}	8.55±0.01^{Cde}	8.19±0.01^{De}	<2.00±0.00^{Bb}
	6	6.51±0.03^{Af}	2.45±0.06^{Be}	8.64±0.03^{Ce}	8.76±0.00^{Df}	2.10±0.03^{Ec}
Anaerobic reducing sulphite *Clostridium* spp. count (log MPN/g)	**0**	<1.47±0.00^{Aa}	<1.47±0.00^{Aa}	<1.47±0.00^{Aa}	<1.47±0.00^{Aa}	<1.47±0.00^{Aa}
	1	1.60±0.00^{Ab}	<1.47±0.00^{Ba}	<1.47±0.00^{Ba}	2.30±0.00^{Cb}	<1.47±0.00^{Ba}
	2	2.04±0.00^{Ac}	<1.47±0.00^{Ba}	<1.47±0.00^{Ba}	3.17±0.00^{Cc}	<1.47±0.00^{Ba}
	3	<1.47±0.00^{Aa}	1.47±0.00^{Aa}	3.17±0.00^{Bb}	>4.38±0.00^{Cd}	<1.47±0.00^{Aa}
	4	<2.47±0.00^{Ad}	<1.47±0.00^{Ba}	3.44±0.00^{Cc}	5.04±0.00^{De}	<1.47±0.00^{Ba}
	5	2.60±0.00^{Ae}	<1.47±0.00^{Ba}	2.84±0.00^{Cd}	2.47±0.00^{Df}	<1.47±0.00^{Ba}
	6	<2.47±0.00^{Ad}	<1.47±0.00^{Ba}	2.60±0.00^{Ce}	<2.47±0.00^{Af}	<1.47±0.00^{Ba}
Total yeast, mold count (log cfu/g)	**0**	<1.00±0.00^{Aa}	<1.00±0.00^{Aa}	<1.00±0.00^{Aa}	<1.00±0.00^{Aa}	<1.00±0.00^{Aa}
	1	3.90±0.02^{Ab}	<1.00±0.00^{Ba}	3.99±0.04^{Ab}	2.09±0.39^{Cb}	<1.00±0.00^{Ba}
	2	5.08±0.04^{Ac}	<1.00±0.00^{Ba}	4.85±0.05^{Cc}	4.60±0.00^{Dc}	<1.00±0.00^{Ba}
	3	5.95±0.00^{Ad}	<1.00±0.00^{Ba}	5.32±0.03^{Cd}	5.27±0.02^{Dd}	<1.00±0.00^{Ba}
	4	6.08±0.02^{Ad}	<1.00±0.00^{Ba}	5.78±0.33^{Ae}	5.94±0.04^{Ae}	<1.00±0.00^{Ba}
	5	6.70±0.08^{Ae}	<1.00±0.00^{Ba}	5.93±0.12^{Ce}	6.25±0.04^{De}	<1.00±0.00^{Ba}
	6	5.57±0.27^{Af}	<1.00±0.00^{Ba}	6.04±0.04^{Ce}	7.25±0.01^{Df}	<1.00±0.00^{Ba}

All values are the mean ± standard deviation (n. = 3).
Different letters (A, B, C) in the same line indicate significant differences (P<0.05).
Different letters (a, b, c) in the same column indicate significant differences (P<0.05).

and psychrotrophic bacteria counts in all group samples according to the length of storage. The maximum level of acceptability of TAC in fish products has not, in practice, the reported level of mesophilic aerobic bacteria valid for these bacteria (BELL *et al.*, 2005). In this study, the limit of acceptability in terms of TAC was 2 weeks for WPI, 3 weeks for EP, 4 weeks for C and G samples. There is very little published data about the TAC levels of hot smoked vacuum packed fish products. CAKLI *et al.* (2006) found that the TAC was 4.9 log cfu/g in hot smoked rainbow trout (*Oncorhynchus mykiss*) samples stored in a vacuum pack for 40 days at 4°C. The most important microbiological risk is reported in the smoked fish products of *Clostridium* spp. (ESPE *et al.*, 2004). In this study, the anaerobic reducing sulphite *Clostridium* spp. count was determined as <1.47±0.00 log MNP/g in all analysed groups at initial storage. While the anaerobic reducing sulphite *Clostridium* spp. count of the control group increased to 2.60±0.00 log MNP/g value in the 5th week of storage, it rose to 3.44±0.00 log MNP/g in the WPI group and to 5.04±0.00 log MNP/g in the EP group in the 4th week of storage, and to 2.84±0.00 log MNP/g in the G group in the 6th week of storage. The 1.60±0.00 log MNP/g value was found in the Co group in the 1st week of storage, in the AM group in the 3rd week of storage, and in subsequent weeks was established at a value of <1.47±0.00 log MNP/g. *Clostridium perfringens* and *Clostridium botulinum* has not been determined in all groups during storage.

Table 1 (followed) - Microbiological parameters of hot smoked rainbow trout fillets coated with protein based film during storage at 2°±2°C.

	Storage time (week)	Z	G	Co	RT	AM
Total mesophilic bacteria count (log cfu/g)	**0**	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}
	1	<1.00±0.00^{Ba}	2.91±0.03^{Db}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}
	2	<1.00±0.00^{Ba}	3.89±0.07^{Ec}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}
	3	<1.00±0.00^{Ea}	4.25±0.09^{Fd}	<1.00±0.00^{Ea}	<1.00±0.00^{Ea}	<1.00±0.00^{Ea}
	4	<1.00±0.00^{Ba}	**7.57±0.02^{Ae}**	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}
	5	<1.00±0.00^{Ba}	8.06±0.06^{Af}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}
	6	<1.00±0.00^{Da}	8.06±0.06^{Ef}	<1.00±0.00^{Da}	<1.00±0.00^{Da}	1.00±0.00^{Da}
Total psychrotrophic bacteria count (log cfu/g)	**0**	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}
	1	<1.00±0.00^{Ba}	3.99±0.12^{Db}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}
	2	<1.00±0.00^{Ba}	4.99±0.06^{Ec}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}
	3	<1.00±0.00^{Da}	5.19±0.05^{Ed}	<1.00±0.00^{Da}	<1.00±0.00^{Da}	<1.00±0.00^{Da}
	4	<1.00±0.00^{Ea}	**6.78±0.03^{Fe}**	<1.00±0.00^{Ea}	<1.00±0.00^{Ea}	<1.00±0.00^{Ea}
	5	<1.00±0.00^{Ea}	8.11±0.00^{Ff}	<1.00±0.00^{Ea}	<1.00±0.00^{Ea}	<1.00±0.00^{Ea}
	6	<1.00±0.00^{Ea}	8.14±0.05^{Ff}	<1.00±0.00^{Ea}	<1.00±0.00^{Ea}	<1.00±0.00^{Ea}
Total anaerobic bacteria count (log cfu/g)	**0**	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}
	1	<1.00±0.00^{Ba}	3.73±0.03^{Eb}	<2.00±0.00^{Fb}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}
	2	<1.00±0.00^{Ba}	4.71±0.08^{Ec}	<2.00±0.00^{Fb}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}
	3	<1.00±0.00^{Fa}	4.84±0.10^{Gd}	2.47±0.01^{Hc}	<1.00±0.00^{Fa}	<1.00±0.00^{Fa}
	4	<1.00±0.00^{Ea}	**6.69±0.00^{Ae}**	2.60±0.06^{Fcd}	<1.00±0.00^{Ea}	<1.00±0.00^{Ea}
	5	<1.00±0.00^{Ea}	8.11±0.00^{Ff}	<2.00±0.00^{Bb}	<1.00±0.00^{Ea}	<1.00±0.00^{Ea}
	6	<1.00±0.00^{Fa}	8.04±0.03^{Gfg}	<1.00±0.00^{Fa}	<1.00±0.00^{Fa}	<1.00±0.00^{Fa}
Anaerobic reducing sulphite *Clostridium* spp. count (log MPN/g)	**0**	<1.47±0.00^{Aa}	<1.47±0.00^{Aa}	<1.47±0.00^{Aa}	<1.47±0.00^{Aa}	<1.47±0.00^{Aa}
	1	<1.47±0.00^{Ba}	2.04±0.00^{Db}	1.60±0.00^{Ab}	<1.47±0.00^{Ba}	<1.47±0.00^{Ba}
	2	<1.47±0.00^{Ba}	2.44±0.00^{Dc}	<1.47±0.00^{Ba}	<1.47±0.00^{Ba}	<1.47±0.00^{Ba}
	3	<1.47±0.00^{Aa}	2.47±0.00^{Dd}	<1.47±0.00^{Aa}	<1.47±0.00^{Aa}	1.60±0.00^{Eb}
	4	<1.47±0.00^{Ba}	<2.47±0.00^{Acd}	<1.47±0.00^{Ba}	<1.47±0.00^{Ba}	<1.47±0.00^{Ba}
	5	<1.47±0.00^{Ba}	2.60±0.00^{Ae}	<1.47±0.00^{Ba}	<1.47±0.00^{Ba}	<1.47±0.00^{Ba}
	6	<1.47±0.00^{Ba}	2.84±0.00^{Df}	<1.47±0.00^{Ba}	<1.47±0.00^{Ba}	<1.47±0.00^{Ba}
Total yeast, mold count (log cfu/g)	**0**	<1.00±0.00^{Aa}	<1.00±0.00^{Aa}	<1.00±0.00^{Aa}	<1.00±0.00^{Aa}	<1.00±0.00^{Aa}
	1	<1.00±0.00^{Ba}	2.20±0.50^{Cb}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}
	2	<1.00±0.00^{Ba}	3.67±0.23^{Ec}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}
	3	<1.00±0.00^{Ba}	4.83±0.00^{Ed}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}
	4	<1.00±0.00^{Ba}	5.27±0.04^{Cde}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}
	5	<1.00±0.00^{Ba}	5.26±0.02^{Ede}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}
	6	<1.00±0.00^{Ba}	5.50±0.02^{Aef}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}	<1.00±0.00^{Ba}

All values are the mean ± standard deviation (n. = 3).
Different letters (A, B, C) in the same line indicate significant differences (P<0.05).
Different letters (a, b, c) in the same column indicate significant differences (P<0.05).

The mold and yeast load of hot smoked fish used in this study was good, as indicated by a low initial number of bacteria (<1 log cfu/g). Table 1 shows an increase in the number of the mold and yeast for the control, WPI, EP and G groups. The yeast-mold load was found to be 5.75±0.10 log cfu/g in the C samples, 5.70±0.15 log cfu/g in the WPI samples, 6.84±0.03 log cfu/g in the EP samples and 5.07±0.09 log cfu/g in the G samples in the 8th week of storage.

The results of the sensory assessment of samples are given in Table 2. The fish samples were considered to be acceptable for human consumption until the sensory score reached 5 (MOHAN *et al.*, 2012). Overall acceptability of the control, SPI, WPI, EP, WG, Z, TR and AM group samples were given 'unacceptable' scores by the 4th week. Based on sensory data, the shelf life of G and Co samples stored under refrigeration was 5 weeks. The shelf life of hot smoked bonito and anchovy stored at 4°C were reported to be 10 and 11 days (KORAL and KOSE, 2005; KORAL *et al.*, 2009). The shelf-life of hot-smoked bonito wrapped in stretch film and stored in a refrigerator was determined as 2 weeks (KAYA *et al.*, 2006), and the shelf-life of tilapia under the same conditions was determined to be 3 weeks (YANAR, 2007).

In another study, the shelf-life of hot-smoked vacuum packed rainbow trouts was specified as 4 weeks (ERKAN *et al.*, 2009; ERKAN *et al.*, 2011a). In this study, the protein-based coating process (with the exception of G and Co groups) was not effective on the sensory shelf-life of the products. Glacial acetic acid and NaOH as pH regulators used in the preparation of the coating material reduced scores given to the taste and smell of the product. Other chemicals such as pH regulators can be tested in accordance with the flavor of the food. However, in another study Chitosan-coated samples (salted and dried horse mackerel) received higher overall consumer acceptance compared with the control group (AHN and LEE, 1992).

The results of *L**, *a** and *b** values are summarized in Table 3. *L** values of the control, WG and Z group samples decreased, and the *a** value of the WG group and the *b** value of the C, SPI and Z group samples increased. The differences in the amount of WP, EP, AM groups were not found to be significant (P>0.05) on *L**, *a** and *b** values. SATHIVEL (2005) reported that the effects of coating with egg albumen, soy protein concentrate, pink salmon protein, arrowtooth flounder protein and chitosan on *a*, *b* and whiteness values of cooked pink salmon fillets were not significant. CHIDANANDAIAH *et al.* (2009) reported that sodium alginate coating improved the sensory quality of beef meat patties in refrigerated storage.

Table 2 - Sensory parameters of hot smoked rainbow trout fillets coated with protein based film during storage at 2°±2°C.

	Storage time (week)	C	SPI	WPI	EP	WG
General average of sensory parameters	**0**	8.20±0.42	8.28±0.47	7.69±0.57	7.41±0.31	6.74±0.43
	1	7.20±0.23	7.47±0.10	6.75±0.23	6.97±0.09	6.08±0.34
	2	6.78±0.35	7.26±0.06	6.35±0.30	6.05±0.22	5.92±0.34
	3	**5.45±0.56**	**6.05±0.15**	**5.35±0.11**	**5.14±0.29**	**5.04±0.24**
	4	4.43±0.29	4.53±0.13	3.88±0.24	4.48±0.13	3.50±0.52
	5	-	-	-	-	-
	6	-	-	-	-	
Consumability time	**General appearance**	3 weeks	3 weeks	3 weeks	2 weeks	3 weeks
	Odor	3 weeks	3 weeks	3 weeks	3 weeks	3 weeks
	Taste	2 weeks	3 weeks	3 weeks	3 weeks	2 weeks
	Tissue	3 weeks	3 weeks	3 weeks	3 weeks	3 weeks
	Average	**3 weeks**	**3 weeks**	**3 weeks**	**3 weeks**	**3 weeks**
	Storage time (week)	**Z**	**G**	**Co**	**RT**	**AM**
General average of sensory parameters	**0**	6.84±0.51	7.97±0.12	7.74±0.29	7.08±0.68	7.28±0.33
	1	6.08±0.35	6.58±0.15	7.40±0.29	6.28±0.33	6.85±0.15
	2	5.89±0.39	6.35±0.17	6.70±0.22	5.64±0.26	6.23±0.38
	3	**5.17±0.44**	6.01±0.15	5.59±0.30	**5.00±0.26**	**5.38±0.47**
	4	4.33±0.45	5.78±0.56	5.39±0.22	3.73±0.04	4.23±0.29
	5	-	**5.10±0.41**	**5.10±0.43**	-	-
	6	-4.45±0.46	4.15±0.59	-	-	
Consumability time	**General appearance**	3 weeks	5 weeks	5 weeks	3 weeks	3 weeks
	Odor	2 weeks	5 weeks	5 weeks	2 weeks	3 weeks
	Taste	2 weeks	3 weeks	4 weeks	2 weeks	2 weeks
	Tissue	3 weeks	4 weeks	5 weeks	3 weeks	3 weeks
	Average	**3 weeks**	**5 weeks**	**5 weeks**	**3 weeks**	**3 weeks**

Table 3 - Colour parameters of hot smoked rainbow trout fillets coated with protein based film during storage at 2°±2°C.

Edible protein film		C	SPI	WP	EP	WG
*L**	First measurement	73.09±0.97^{Aa}	61.23±1.20^{Ba}	73.80±0.81^{ACa}	75.67±0.47^{Ca}	73.23±0.47^{Aa}
	Second measurement	70.23±0.06^{AFb}	62.67±1.19^{Ba}	73.64±0.09^{CDa}	75.01±0.35^{DGa}	72.14±0.33^{ACb}
*a**	First measurement	2.80±042^{Aa}	10.82±0.38^{Ba}	3.72±0.89^{ACa}	1.51±0.41^{Aa}	1.94±0.10^{Aa}
	Second measurement	3.05±0.67^{ACa}	9.99±0.58^{Ba}	3.16±0.47^{ACa}	1.23±0.81^{Aa}	2.35±0.14^{ACb}
*b**	First measurement	20.03±0.09^{ACa}	19.38±0.62^{ACa}	22.03±1.06^{Aba}	20.39±0.89^{ACa}	24.54±1.76^{Ba}
	Second measurement	22.44±0.72^{Ab}	20.45±0.22^{Ab}	22.88±2.09^{Aa}	21.15±0.85^{Aa}	22.68±0.32^{Aa}
Δ*E**		3.84±0.49^{AC}	2.26±0.79^{AB}	2.33±0.46^{AB}	1.58±0.31^{B}	2.23±0.24^{AB}
		Z	**G**	**Co**	**RT**	**AM**
*L**	First measurement	69.31±0.45^{Da}	67.02±0.33^{Da}	64.31±0.09^{Ea}	76.13±0.63^{Ca}	68.03±0.40^{Da}
	Second measurement	66.27±0.89^{Eb}	70.00±0.95^{AFb}	69.54±0.80^{FHb}	77.30±0.18^{Gb}	67.57±0.46^{EHa}
*a**	First measurement	2.00±0.88^{Aa}	1.47±1.15^{Aa}	5.99±1.12^{Ca}	1.68±0.27^{Aa}	3.09±0.32^{Aa}
	Second measurement	1.93±0.62^{ACa}	1.18±0.38^{Aa}	3.52±1.04^{Cb}	1.93±0.27^{ACa}	2.92±0.24^{ACa}
*b**	First measurement	19.37±0.98^{ACa}	18.12±1.54^{Ca}	18.71±0.83^{ACa}	20.45±0.53^{ACa}	20.27±0.95^{ACa}
	Second measurement	22.43±1.05^{Ab}	19.71±0.36^{Aa}	20.99±1.97^{Aa}	20.52±0.51^{Aa}	21.63±0.78^{Aa}
Δ*E**		4.54±0.53^{C}	3.51±0.61^{ACE}	6.61±0.70^{D}	2.48±0.42^{AB}	1.62±0.59^{BE}

All values are the mean ± standard deviation (n. = 3).
Different letters (a, b, c) in the same line indicate significant differences (P<0.05).
Different letters (A, B, C) in the same column indicate significant differences (P<0.05).

The results of the texture profile values are also shown in Table 4. Measurements of the texture profile of hot smoked fish fillets showed that the texture parameters of the protein-based coating process did not generally cause changes. The data could not be ascertained to discuss the parameters of the texture in smoked fish.

The TVB-N values for the treatments during storage are presented in Table 5. The initial TVB-N values (mg N per 100 g of fish) ranged from 9.07 to 16.38 in coated samples. GIMÉNEZ *et al.* (2002) proposed a value of 25 mg N/100 g flesh as the highest acceptable level for trout. In our study, in the WG, Z, RT and AM samples the TVB-N values remained below this limit of acceptability throughout the entire storage period. The TVB-N value limit was exceeded in the EP group in the 2nd week of storage, in the G and the control group in the 3rd week of storage, in the WPI group in the 4th week of storage, in the SPI group in the 5th week of storage, and in the Co group in the 6th week of storage.

Using cod and herring fillets and different types of soluble chitosan coatings, JEON *et al.* (2002) reported a reduction of 33–50% and 26–51%, respectively, in TVB-N formation at the end of a 12-day storage period. LÓPEZ-CABALLERO *et al.* (2005) found the protective chitosan–gelatin coating distinctly lowered TVB-N values and hence slowed spoilage. Hydroperoxides and secondary substances (carbonyl group secondary substances formed by oxidation of hydroperoxides) causes the loss of taste and odor substances specific to food products. TBA-i is also a measure of MDA and is the secondary/last product of lipid oxidation (GOULAS and KONTOMINAS, 2005). In this study, the TBA-i values of the Z group lower than the other groups, whilst the TBA-i values of the RT and AM groups remained higher than the other groups during storage. In general, the limit value of 2 mg (GOULAS and KONTOMINAS, 2005) is not exceeded by all groups during storage. It was observed in the literature that edible films decreased the TBA-i value of coated seafoods. For example, perch and salmon treated with Flavor-Tex alginate coatings and placed into PE bags obtained slightly lower lipid oxidation products than the uncoated controls placed in the PE bags (GENNADIOS *et al.*, 1997).

4. CONCLUSIONS

The storage of hot smoked rainbow trout coated with SPI, WG, Z, Co, RT, AM and under vacuum packaged conditions inhibited bacterial growth, reduced the formation TVB-N and TBA-i, and extended the shelf life. Thanks to their physicochemical properties gained from contained acetic acid (in WG, Z, Co, RP and AB groups), alcohol (in Z group) or isoflavones (in the SPI group), they have a higher antimicrobial activity and thus a prolongation of shelf-

Table 4 - Texture parameters of hot smoked rainbow trout fillets coated with protein based film during storage at 2°±2°C.

	Texture parameters			
	Fracturability (N)		Hardness (N)	
Edible protein film	First measurement	Second measurement	First measurement	Second measurement
C	1.933±0.074^{Aa}	1.749±0.199^{ACa}	1.601±0.100^{Aba}	1.363±0.117^{ACb}
SPI	1.343±0.063^{ABa}	1.529±0.157^{Aa}	1.119±0.035^{ABa}	1.266±0.144^{Aa}
WP	1.869±0.311^{ACa}	3.157±0.357^{Bb}	1.825±0.573^{Aa}	2.484±0.358^{Ba}
EP	1.155±0.268^{Ba}	1.585±0.414^{Ab}	0.955±0.257^{Ba}	1.247±0.410^{Aa}
WG	1.366±0.278^{ABa}	1.493±0.370^{Aa}	1.072±0.219^{ABa}	1.227±0.370^{Aa}
Z	1.591±0.063^{ABa}	2.549±0.075^{BCb}	1.232±0.134^{ABa}	2.241±0.096^{BCb}
G	1.229±0.189^{CBa}	0.921±0.154^{Aa}	1.025±0.177^{ABa}	0.729±0.136^{Aa}
Co	1.405±0.269^{ABa}	1.339±0.261^{Aa}	1.155±0.238^{ABa}	1.069±0.278^{Aa}
RT	1.249±0.073^{ABa}	1.321±0.306^{Aa}	1.059±0.033^{ABa}	1.057±0.298^{Aa}
AM	1.170±0.128^{BCa}	1.185±0.101^{Aa}	0.980±0.082^{Ba}	0.917±0.053^{Aa}
	Gumminess (N)		Chewiness (N×mm)	
	First measurement	Second measurement	First measurement	Second measurement
C	0.731±0.133^{Aa}	0.546±0.028^{Ab}	1.63±0.40^{Aa}	1.09±0.01^{Ab}
SPI	0.530±0.041^{Aa}	0.588±0.054^{Aa}	1.88±0.73^{Aa}	1.67±0.25^{ACa}
WP	0.817±0.214^{Aa}	1.098±0.190^{Ba}	1.51±0.15^{Aa}	3.24±0.10^{BDb}
EP	0.455±0.146^{Aa}	0.525±0.175^{Aa}	1.13±0.29^{Aa}	1.65±0.44^{ACa}
WG	0.489±0.111^{Aa}	0.534±0.186^{Aa}	1.09±0.23^{Aa}	1.32±0.50^{Aa}
Z	0.544±0.111^{Aa}	1.063±0.062^{Bb}	1.44±0.47^{Aa}	2.63±0.23^{BCb}
G	0.425±0.091^{Aa}	0.334±0.048^{Aa}	1.24±0.48^{Aa}	4.15±0.51^{Db}
Co	0.528±0.132^{Aa}	0.478±0.130^{Aa}	1.22±0.35^{Aa}	1.17±0.36^{Aa}
RT	0.514±0.007^{Aa}	0.482±0.141^{Aa}	1.20±0.09^{Aa}	1.25±0.23^{Aa}
AM	0.431±0.024^{Aa}	0.343±0.020^{Ab}	0.90±0.04^{Aa}	0.64±0.08^{Ab}
	Springiness (mm)		Adhesiveness (N×mm)	
Edible protein film	First measurement	Second measurement	First measurement	Second measurement
C	2.25±0.17^{Aa}	2.04±0.10^{Aa}	0.06±0.04^{Aa}	0.06±0.00^{ABa}
SPI	2.50±0.05^{Aa}	2.89±0.17^{ABb}	0.04±0.01^{Aa}	0.04±0.02^{ABa}
WP	2.82±0.71^{Aa}	2.84±0.20^{Aba}	0.05±0.02^{Aa}	0.11±0.04^{Aa}
EP	2.20±0.05^{Aa}	4.43±0.94^{Bb}	0.04±0.01^{Aa}	0.05±0.00^{ABa}
WG	2.46±0.19^{Aa}	2.45±0.40^{Aa}	0.07±0.03^{Aa}	0.05±0.03^{ABa}
Z	2.62±0.41^{Aa}	2.52±0.17^{Aa}	0.04±0.02^{Aa}	0.05±0.00^{ABa}
G	3.14±0.80^{Aa}	2.15±0.64^{Ab}	0.08±0.02^{Aa}	0.03±0.01^{Bb}
Co	2.34±0.16^{Aa}	1.99±0.51^{Aa}	0.05±0.02^{Aa}	0.02±0.00^{Bb}
RT	2.28±0.23^{Aa}	2.93±0.89^{ABa}	0.03±0.02^{Aa}	0.04±0.02^{ABa}
AM	2.12±0.09^{Aa}	1.91±0.15^{Aa}	0.09±0.03^{Aa}	0.04±0.01^{ABb}
	Cohesiveness		Resilience	
	First measurement	Second measurement	First measurement	Second measurement
C	0.38±0.07^{Aa}	0.31±0.02^{ACa}	0.11±0.03^{Aa}	0.08±0.01^{Aa}
SPI	0.40±0.01^{Aa}	0.39±0.01^{ABa}	0.12±0.01^{Aa}	0.11±0.01^{ABa}
WP	0.41±0.02^{Aa}	0.35±0.03^{ABCb}	0.12±0.04^{Aa}	0.10±0.02^{ABa}
EP	0.39±0.03^{Aa}	0.33±0.03^{ACa}	0.11±0.02^{Aa}	0.10±0.00^{ABa}
WG	0.36±0.01^{Aa}	0.35±0.04^{ABCa}	0.10±0.02^{Aa}	0.10±0.00^{ABa}
Z	0.34±0.07^{Aa}	0.42±0.01^{Ba}	0.10±0.03^{Aa}	0.13±0.00^{Ba}
G	0.34±0.02^{Aa}	0.36±0.01^{ABCa}	0.09±0.02^{Aa}	0.10±0.01^{ABa}
Co	0.37±0.02^{Aa}	0.35±0.03^{ABCa}	0.11±0.01^{Aa}	0.10±0.01^{ABa}
RT	0.41±0.03^{Aa}	0.36±0.02^{ABCa}	0.12±0.01^{Aa}	0.09±0.02^{ABa}
AM	0.37±0.02^{Aa}	0.29±0.01^{Cb}	0.10±0.02^{Aa}	0.08±0.00^{Aa}

All values are the mean ± standard deviation (n. = 3)
Different letters (a, b, c) in the same line indicate significant differences (P<0.05).
Different letters (A, B, C) in the same column indicate significant differences (P<0.05).

Table 5 - Chemical parameters of hot smoked rainbow trout fillets coated with protein based film during storage at 2°±2°C.

	Storage time (week)	C	SPI	WPI	EP	WG
TVB-N value (mg/100 g)	**0**	15.28±0.05^{Aab}	16.38±0.49^{Aa}	14.42±0.19^{Ba}	11.85±0.31^{Ca}	12.13±0.54^{Ca}
	1	21.11±0.91^{Ab}	17.11±0.55^{BCa}	17.71±0.78^{Bb}	16.33±0.96^{BCDb}	15.66±0.22^{BCDb}
	2	21.28±0.62^{ABb}	21.95±0.25^{Ab}	19.95±0.17^{BEc}	25.76±0.96^{Cc}	15.49±0.51^{DGb}
	3	27.68±0.26^{AEc}	23.24±0.18^{Bbc}	22.87±0.54^{Bd}	27.99±0.55^{Ac}	16.72±0.56^{Cbce}
	4	27.39±0.75^{Acd}	24.53±0.29^{Bcd}	25.08±0.36^{Be}	22.34±0.96^{Cd}	16.03±0.27^{Dbc}
	5	25.63±0.49^{Ade}	25.20±0.31^{Ad}	27.88±0.50^{Bf}	19.96±0.62^{CFe}	17.52±0.34^{Dcd}
	6	24.46±0.49^{Ae}	20.38±0.73^{BCe}	21.57±0.41^{BDcd}	20.94±0.50^{BDde}	19.02±0.57^{Cd}
TBA-i value (mg MDA/kg)	**0**	0.47±0.00^{Aa}	0.00±0.00^{Ba}	0.00±0.00^{Ba}	0.00±0.00^{Ba}	0.00±0.00^{Ba}
	1	0.74±0.03^{Abd}	1.56±0.24^{Bb}	0.91±0.01^{Cb}	0.55±0.01^{Db}	1.10±0.05^{EGb}
	2	0.52±0.01^{Aa}	0.54±0.02^{Ac}	0.88±0.03^{Bb}	0.42±0.01^{Cc}	0.74±0.03^{Dc}
	3	1.23±0.09^{ACFc}	1.06±0.01^{BDd}	1.14±0.03^{BCFc}	0.98±0.01^{Dd}	1.32±0.03^{AHd}
	4	0.87±0.06^{Ab}	1.15±0.07^{BCde}	1.05±0.05^{CFc}	0.74±0.03^{ADe}	1.63±0.05^{Ee}
	5	0.78±0.02^{Abd}	1.26±0.04^{Bbe}	1.47±0.04^{Cd}	1.11±0.05^{DGf}	0.89±0.01^{Ef}
	6	0.89±0.02^{Ab}	1.71±0.01^{Bf}	1.10±0.01^{Cc}	1.07±0.02^{Cf}	1.05±0.03^{Cb}
	Storage time (week)	**Z**	**G**	**Co**	**RT**	**AM**
TVB-N value (mg/100 g)	**0**	13.99±0.57^{Ba}	9.33±0.18^{Da}	9.74±0.47^{Da}	9.07±0.25^{Da}	10.22±0.73^{Da}
	1	17.50±0.32^{Bb}	17.13±0.68^{BCbe}	15.48±0.84^{BCDb}	14.78±0.50^{CDb}	14.52±0.55^{Db}
	2	18.78±0.61^{EFc}	19.96±0.41^{BEc}	17.38±0.27^{FHc}	15.84±0.53^{GHb}	16.25±0.24^{DHc}
	3	17.31±0.31^{CDc}	26.00±0.76^{Ed}	18.56±0.19^{Dcf}	18.48±0.85^{Dcf}	18.99±0.09^{Dde}
	4	18.36±0.30^{Ec}	25.11±0.20^{Bd}	23.53±0.31^{BCd}	18.75±0.67^{Ec}	19.31±0.28^{Ede}
	5	20.95±0.59^{CEd}	24.73±0.63^{Ad}	24.46±0.72^{Ad}	21.96±0.77^{Ed}	18.31±0.31^{DFd}
	6	22.52±0.79^{Dd}	24.44±0.22^{Ad}	25.06±0.28^{Ad}	24.50±0.04^{Ae}	18.33±0.16^{Cd}
TBA-i value (mg MDA/kg)	**0**	0.25±0.02^{Ca}	1.22±0.03^{Dac}	1.87±0.04^{Ea}	1.74±0.04^{Fa}	1.18±0.03^{Ga}
	1	0.33±0.01^{Fab}	0.99±0.01^{Gb}	0.93±0.06^{Cb}	1.27±0.07^{BHbd}	1.19±0.01^{EHb}
	2	0.39±0.01^{Cb}	0.95±0.02^{Bb}	1.34±0.03^{Ec}	1.10±0.02^{Fb}	1.61±0.04^{Gc}
	3	0.53±0.01^{Ec}	1.20±0.01^{ABFac}	0.75±0.02^{Gd}	1.24±0.11^{FHbd}	1.38±0.01^{Hd}
	4	0.68±0.04^{Dd}	1.09±0.02^{BFbc}	0.87±0.02^{AGbd}	1.47±0.01^{Ec}	1.22±0.06^{BHb}
	5	0.53±0.00^{Fc}	0.94±0.001^{Eb}	1.15±0.02^{DGe}	1.20±0.03^{DBbd}	1.07±0.03^{Ge}
	6	1.66±0.01^{Be}	2.08±0.07^{Dd}	1.11±0.02^{Cef}	1.23±0.03^{Ebd}	1.88±0.01^{Ff}

All values are the mean ± standard deviation (n. = 3)
Different letters (A, B, C) in the same line indicate significant differences (P<0.05).
Different letters (a, b, c) in the same column indicate significant differences (P<0.05).

life by becoming safer in terms of chemical and sensory parameters. Based on the microbiological and sensorial indices, vacuum packaging in combination with the Co protein coating was the most effective treatment for preservation and in extending the shelf life of hot smoked rainbow trout fillets.

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ABBREVIATIONS

AM: Atlantic mackerel
C: Control
Co: Collagen
EP: Egg white powder protein
G: Gelatin
MDA: Malondialdehyde
MPN: Most Probably Number
NaOH: Sodium hydroxide
PCA: Plate count agar
RH: Relative humidity
RT: Rainbow trout
SPI: Soy protein isolates
TAC: Total anaerobe counts
TBA-I:Thiobarbituric acid index
TVB-N: Total volatile basic nitrogen
TVC: Total viable counts
WG: Wheat gluten
WPI: Whey protein isolate
Z: Zein

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PAPER

INFLUENCE OF OZONATED WATER ON MICROBIAL LOAD AND SHELF LIFE OF SHREDDED CARROTS

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ABSTRACT

Efficacy of ozonated water in reducing the counts of *Salmonella* Typhimurium, total mesophilic aerobic bacteria (TMAB) and mould-yeast on carrot and in improving its storage quality was tested. Treatment of carrots with ozonated water (0.5, 1.0 and 1.5 ppm) for 3, 5 and 10 min caused reductions in the number of TMAB, mould–yeast and *Salmonella* Typhimurium ranging between 0.09-1.1, 0.77-0.96 and 0.37-2.72 log units, respectively. Tap water was used as control treatment. Under storage conditions at 4°C for 15 days, TMAB and mould-yeast counts of ozone treated samples were found significantly lower than control samples (p<0.05).

- Keywords: carrot, decontamination, ozonated water, shelf life, *S.* Typhimurium -

INTRODUCTION

There has been an increasing demand all over the world to consume fresh vegetables and fruits for health and nutritional reasons. As a result, outbreak reports related to fresh produces increased in recent years (ALEXOPOULOS *et al.*, 2013). Various bacterial pathogens were reported to survive and grow on these types of products and *Salmonella* is one of the most frequently reported cause of foodborne outbreaks for fresh vegetables and fruits (CDC, 2012).

Sanitization of fresh produce plays an important role in ensuring the food safety and chlorine is the most widely used disinfectant in removing pathogens from vegetables and fruits (ADAMS *et al.*, 1989; LIN *et al.*, 2000; BURNETT and BEUCHAT, 2002; SINGH *et al.*, 2002). The efficiency of numerous chemical and physical methods for assuring the microbiological safety of fresh-cut produce has been covered in several reviews (PARISH *et al.*, 2003; SAPERS, 2003; ALLENDE *et al.*, 2006; RICO *et al.*, 2007; ARTES *et al.*, 2009; GIL *et al.*, 2009; GOMEZ-LOPEZ *et al.*, 2009; ÖLMEZ and KRETZSCHMAR, 2009). On the other hand, consumers should be aware about the residual presence of dangerous by-products from chlorine and other chemicals (ÖLMEZ and KRETZSCHMAR, 2009).

The interest in ozone as an alternative to chlorine and chemical disinfectants is based on its high biocidal efficacy, wide antimicrobial spectrum, absence of by-products, and the ability to generate it when needed (PASCUAL *et al.*, 2007). Because ozone has proven to be a useful antimicrobial, the United States Food and Drug Administration (FDA) amended the food additive regulations to allow the use of gaseous and/or aqueous ozone as an antimicrobial treatment for foods (USDA, 1997). Ozonated water has been tested for its efficacy in the decontamination of fresh produce (KIM *et al.*, 1999; SHARMA *et al.*, 2002a,b; SINGH *et al.*, 2002; SINGH *et al.*, 2003; WADE *et al.*, 2003; KETTERINGHAM *et al.*, 2006; HILDEBRAND *et al.*, 2008; ALEGRIA *et al.*, 2009; CHAUHAN *et al.*, 2011; DING *et al.*, 2011; ALEXOPOULOS *et al.*, 2013). It has been reported that ozonated water effectively kill spoilage microorganisms (*Alicyclobacillus acidocaldarius, Pseudomonas aeruginosa, Zygosaccharomyces bailii*), environmental and fecal contaminants (*Enterococcus faecalis, Escherichia coli*), and food-borne pathogens (*Listeria monocytogenes, Bacillus cereus, Salmonella* Typhimurium, *Yersinia enterocolitica, Staphylococcus aureus*) in low ozone demand media (RESTAINO *et al.*, 1995; KHADRE *et al.*, 2001). Moreover, ozone treatment is effective in maintaining the sensorial quality of fresh produce (WANG *et al.*, 2004).

Several commercial ozone generators are currently available that produce both gaseous and ozone dissolved in water. Conventional ozone generators that need compressors, air dryers, oxygen concentrators and ozone injectors, are too bulky to be used, in point-of-installations (RICE, 1996). On the other hand, in ozo-pen system, which is the technology developed in recent years, ozone can be produced directly from the water to be treated itself instead of producing ozone from a dry air or dry oxygen gas source and the system only needs handheld ozone generator, portable container and water (ANONYMOUS, 2012).

Therefore, the objective of the present study was to determine the effects of ozonated water produced by ozo-pen probes on natural microflora, inoculated pathogen (*Salmonella* Typhimurium) and shelf life of carrots.

MATERIALS AND METHODS

Sample preparation

Carrots (*Daucus carota* L.) were purchased from a local supermarket in Izmir, Turkey. All carrots were pre-cleaned, knife-peeled and washed with tap water. Then the carrots were shredded into pieces 2 to 5 mm wide by means of sterile knife. Ten-gram portions were weighed and placed in sterile jars prior to treatment.

Preparation of treatment waters

Tap water was taken from the laboratory fountain and used directly in the analyses as a treatment solution. Plastic container, which has 20 L volume, was filled with tap water and two ozo-pen probes (Ozomax Inc., Quebec Canada) were dipped into water to produce ozonated water. The system was run till the required concentration of ozone was produced. Ozone concentration was measured by ozone test kit (0-2.3 mg/L, Model OZ-2, Cat.No. 20644-00, Hach Lange, Düsseldorf, Germany). Produced ozonated water (0.5, 1.0 and 1.5 ppm) was used immediately in the analyses. Treatment times (3, 5 and 10 min) were selected as short as possible, because it is known that ozone is highly volatile and difficult to maintain in solution. Residual ozone concentration was checked during experiments to verify ozone concentrations used.

Bacterial culture

Salmonella Typhimurium ATCC 13311 was used in the analysis. A stock culture was grown in Tryptone Soya Broth (TSB, pH 7.3±0.2, Oxoid, Basingstoke, Hampshire, England) supplemented with 50 ppm nalidixic acid (Oxoid, Basingstoke, Hampshire, England, TSBN). Stock cultures on Tryptone Soya Agar (TSA, pH 7.3±0.2, Oxoid, Basingstoke, Hampshire, England) supplemented with 50 ppm nalidixic acid (TSAN) were stored at 4°C and cultured for 24 h at 37°C in TSBN.

Procedure for inoculating carrots

Carrots were checked for *Salmonella* presence before using them as analyse sample. Ten milliliters of 10^{-2} dilution, prepared from 24 h TSB culture (containing 10^8 CFU/mL) was transferred into 1 L of 0.1% sterile peptone water (PW, pH 6.3±0.2, Difco, Detroit, MI, USA), so that the final cell number of *S.* Typhimurium in the suspension was approximately 10^4 CFU/mL. Ten grams of carrot portions were dipped into culture suspension for 1 min, and placed on sterile cheesecloth for removing excess liquid and inoculated samples were transferred into sterile jars. To allow for attachment of *S.* Typhimurium cells, jars partly closed with covers were stored at 20°C for 24 h before they were treated.

Treatment of carrots

For assessing the effect of tap water and ozonated water on natural microflora of carrot, uninoculated carrot samples were used, while carrots were inoculated with *S.* Typhimurium for detecting the effects of treatment solutions on specific pathogen. 10 g of carrot sample was transferred into approximately 100 mL of tap water or ozonated water (0.5, 1.0 and 1.5 ppm), sufficient to cover all the sample in the jar and closed jars were agitated by hand for 3, 5 and 10 min at room temperature. After decanting the treatment solutions, enumeration of total mesophilic aerobic bacteria (TMAB) and mould-yeast for uninoculated samples and enumeration of *S.* Typhimurium for inoculated samples were done. In order to determine the effects of ozonated water (1.5 ppm/5min) and tap water on shelf life of uninoculated carrots, non-treated and previously treated samples were stored at 4°C for 15 days and periodic analyses including TMAB and mould-yeast were applied during storage period.

Microbiological analysis

Each sample (10 g) of carrot treated or untreated was transferred in 90 mL of 0.1% PW and homogenized for 2 min with Stomacher (Lab-blender 400, Seward, London, UK). Appropriate 10 fold dilutions of the samples were prepared in PW and plated in duplicate on/in growth media to estimate microbial counts for TMAB, mould-yeast and *Salmonella*.

TMAB count was determined by using pour plate method on Plate Count Agar (PCA, 7.0±0.2, Oxoid CM325) and incubating the plates at a temperature of 35°C for 24-48 h (BAM, 2001a). The counts of mould-yeast were determined on Dichloran Rose Bengal Chloramphenicol agar (DRBC, pH 5.6±0.2, Oxoid CM0727) at 25°C for 3-5 days (BAM, 2001b). Carrot samples, which were used for inoculation experiment, were firstly checked for the presence of *Salmonella* (BAM, 2007). To enumerate *S.* Typhimurium for inoculated carrot samples, serially diluted homogenates were spread plated (0.1 ml in duplicate) on Bismuth Sulphite Agar supplemented with 50 ppm nalidixic acid (BSAN, pH 7.6±0.2, Oxoid CM0201) and plates were incubated at 37°C for 48 h. It is known that sometimes selective media are not suitable for the recovery of injured cells. Therefore, in the preliminary experiments, the recovery potential of BSA in enumerating *S.* Typhimurium for treated samples were compared with TSAN (pH 7.3±0.2, Oxoid). However, no differences were found between the recovery potentials of the media, and BSAN was used for ongoing work. Randomly selected presumptive *S.* Typhimurium colonies were confirmed using biochemical tests [Triple Sugar Iron Agar (TSI, pH 7.4±0.2, Oxoid) and Lysine Iron Agar (LI, pH 6.7±0.2, Oxoid) reactions] and serological tests (BEUCHAT *et al.*, 1998).

Statistical analysis

Means of microbial populations (log CFU/g) from each treatment were calculated from three replications of each experiment. Data were subjected to analysis of variance and Duncan's multiple tests (SPSS 15.0 for Windows pocket program; SPSS Inc., Chicago, USA) to determine if significant differences ($p<0.05$) between mean values.

RESULTS AND DISCUSSION

In the first part of the study, total mesophilic aerobic bacteria (TMAB) count and mould-yeast count of carrots were investigated in order to evaluate the efficacy of ozonated water on natural background microflora of the samples. Ozonated water (0.5, 1.0 and 1.5 ppm for 3, 5 and 10 min) reduced the initial TMAB counts of carrot samples (6.92 log CFU/g) ranging between 0.09-1.1 log CFU/g, while tap water washing reduced the total counts of carrots between 0.59 and 0.76 log CFU/g ($p>0.05$) (Fig. 1). Statistical analysis showed that there were no significant differences between the exposure times and concentrations used ($p>0.05$). Initial mould and yeast counts of carrot samples (4.95 log CFU/g) were reduced between 0.77 and 0.96 log CFU/g by using ozone washing, depending on ozone concentration and treatment time used (Fig. 2). On the other hand, tap water were found significantly lower effect (0.4-0.64 log reduction) than ozonated water in reducing mould and yeast counts of carrots ($p<0.05$).

Similarly, low reduction effect of ozonated water was reported by KETTERINGHAM *et al.* (2006), who observed maximum reductions of 0.66 log CFU/g and 0.72 log CFU/g on aerobic plate counts of green peppers by washing non-ozonated and ozonated waters, respectively. ALEGRIA *et al.* (2009) observed reductions up to 0.4 log

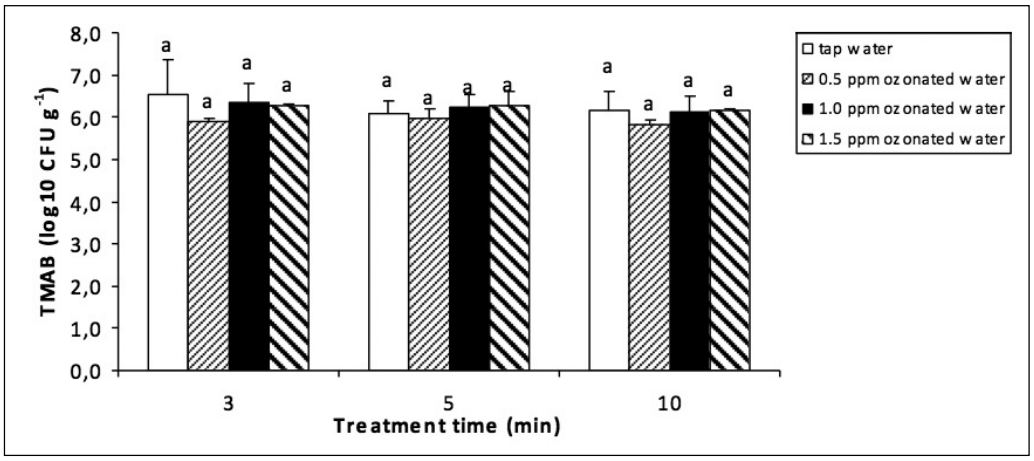


Fig. 1 - Total Mesophilic Aerobic Bacteria (TMAB) counts of carrots treated with tap water and ozonated water at 0.5, 1.0 and 1.5 ppm. Initial TMAB count of untreated carrot sample was 6.92 log CFU/g.
The bars represent standard deviations. Values with different characters, for the same treatment time, differ significantly (p<0.05).

CFU/g in carrot using 1 ppm/5 min. In the other study, treatment of pre-cut carrots with ozonated water (1.3 ppm/2 min) was found insufficient to reduce aerobic plate counts of the samples (KLAIBER *et al.*, 2004). Although these studies showed that ozonated water has limited effect on total aerobic mesophilic bacteria counts of vegetables, there are many studies reporting higher reduction rates obtained by ozonated water (KIM *et al.*, 1999, ACHEN and YOUSEF, 2001, ZHANG *et al.*, 2005, SELMA *et al.*, 2008, ALEXOPOULOS *et al.*, 2013). Maximum 6 log reduction in total mesophilic bacteria of escarole, carrot and spinach was achieved by using different ozone concentrations for 20 min (SELMA *et al.*, 2008). In the same study the investigators observed lower reduction rates (2 and 3 log) for mould and yeast counts of the samples (SELMA *et al.*, 2008). It was reported that pre-cutting or chopping processes may reduce the effect of ozonated water on aerobic plate counts of vegetables (KETTERINGHAM *et al.*, 2006). Moreover, difference in surface area of the vegetables is one of the most important points that affect the decontamination efficacy of ozonated water treatments (GIL *et al.*, 2009; ALEXOPOULOS *et al.*, 2013; SENGUN, 2013). Type and the level of microorganisms are important factors that affect the efficiency of ozone. It was reported that mould-yeast and especially spore forming bacteria require increasing ozone concentrations and longer exposure times for their inactivation (SELMA *et al.*, 2008; ALEXOPOULOS *et al.*, 2013). The solubility, stability, decomposition, concentration and contact time of ozone are also known as other variables that affect the decontamination efficacy of ozone (KIM *et al.*, 1999; RICE, 1999; KETTERINGHAM *et al.*, 2006; BEZIRTZOGLU and ALEXOPOULOS, 2008; ÖLMEZ and AKBAŞ, 2009; BERMUDEZ-AGUIRRE and BARBOSA–CANOVAS, 2013).

In the second part of the study, the impact of water treatments on *S.* Typhimurium in carrot samples was investigated (Fig. 3). Tap water reduced initial count of *S.* Typhimurium (4.67 log CFU/g)

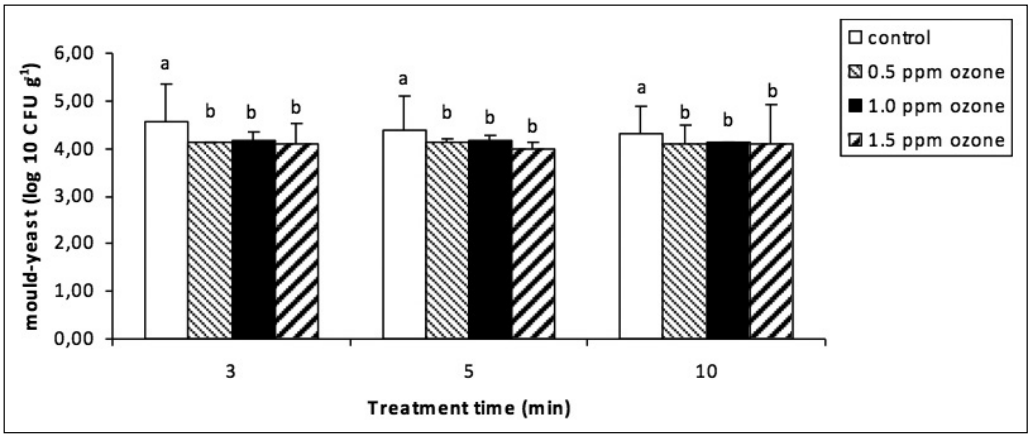


Fig. 2 - Mould and yeast counts of carrots treated with tap water and ozonated water at 0.5, 1.0 and 1.5 ppm. Initial mould and yeast count of untreated carrot sample was 4.95 log CFU/g.
The bars represent standard deviations. Values with different characters, for the same treatment time, differ significantly (p<0.05).

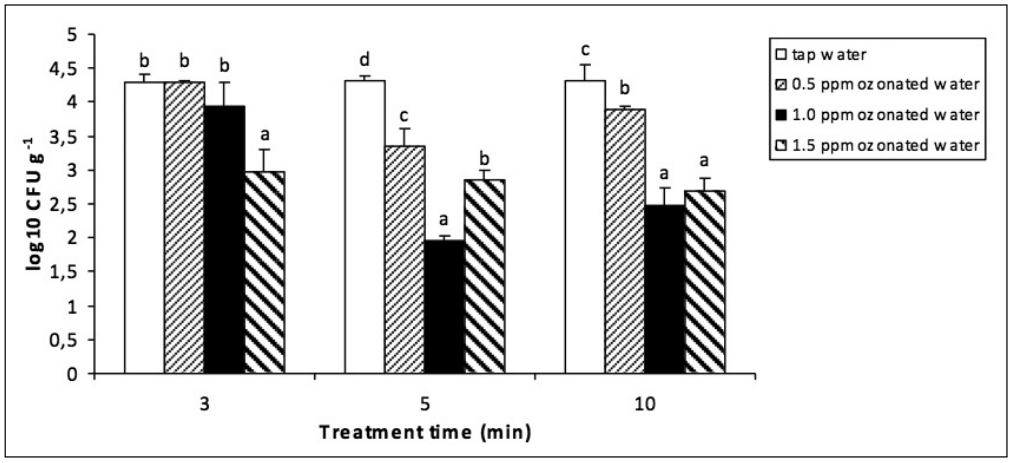


Fig. 3 - S. Typhimurium counts of carrots treated with tap water and ozonated water at 0.5, 1.0 and 1.5 ppm. Initial S. Typhimurium count of untreated carrot sample was 4.67 log CFU/g.
The bars represent standard deviations. Values with different characters, for the same treatment time, differ significantly (p<0.05).

ranging between 0.35-0.39 log CFU/g for 3, 5 and 10 min (p>0.05). On the other hand, ozonated water reduced *S.* Typhimurium counts ranging between 0.37-2.72 log CFU/g, depending on concentration and exposure times used (p<0.05) (Fig. 3). The reduction effect of ozonated water was found significantly different from tap water (p<0.05). Overall interpretation indicated that no significant difference were found between the effects of 1.0 ppm and 1.5 ppm concentrations (p>0.05) for 3, 5 and 10 min exposure times. Therefore 1.5 ppm ozone concentration and 5 min exposure time were chosen as the representative treatment condition for the other steps of this study.

More satisfying results were observed in reducing *S.* Typhimurium than natural microbial load on carrots (Fig. 1, 2 and 3). Similarly, DING *et al.* (2011) investigated the reduction effect of ozonated water (5 mg/L for 3 min dipping) on natural microflora and pathogenic microorganism (*E. coli* O157:H7, *Listeria monocytogenes*, *S.* Typhimurium, *Bacillus cereus*) of oyster mushroom and found 0.612 log CFU/g reduction on total bacteria population and 0.75-1.06 log CFU/g reduction for pathogens. This result could be explained by the different sensitivity of each microorganism to ozone (AKBAS and OZDEMIR, 2008; CULLEN *et al.*, 2010; ALEXOPOULOS *et al.*, 2013).

In the third part of the study, carrot samples, untreated or treated with tap water and ozonated water (1.5 ppm/5 min) were stored at 4°C for 15 days and TMAB and mould-yeast counts were traced during storage period. The initial TMAB count of the untreated carrot (5.51 log CFU/g) was significantly reduced after treatment with tap water (1.26 log CFU/g reduction) and ozonated water (1.79 log CFU/g reduction) (p<0.05) at day 0 and this significant effect was protected till the end of storage (Fig. 4). There were no significant differences between the reduction effects of ozonated water and tap water on TMAB of carrots at day 0, 1, 3 and 5 (p>0.05). On the other hand, TMAB counts of

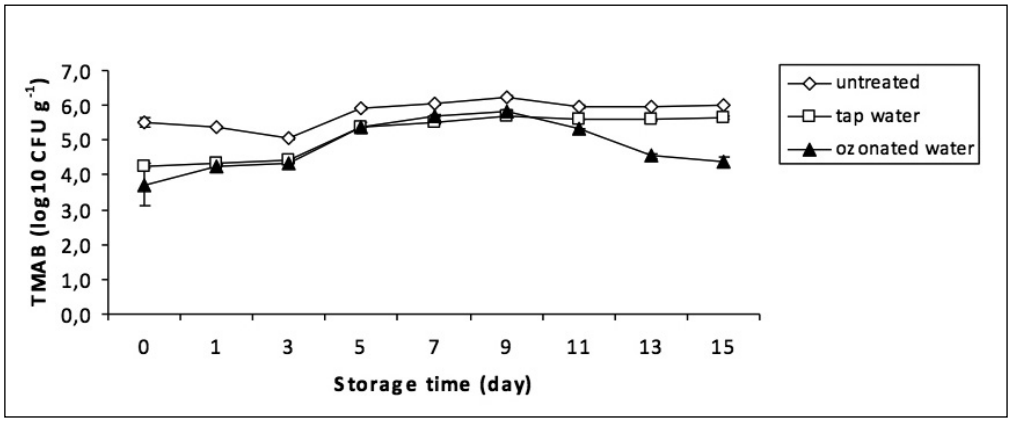


Fig. 4 - Changes in Total Mesophilic Aerobic Bacteria (TMAB) counts of untreated and treated carrots with tap water (5 min) and ozonated water (1.5 ppm /5 min) during storage period.
The bars represent standard deviations.

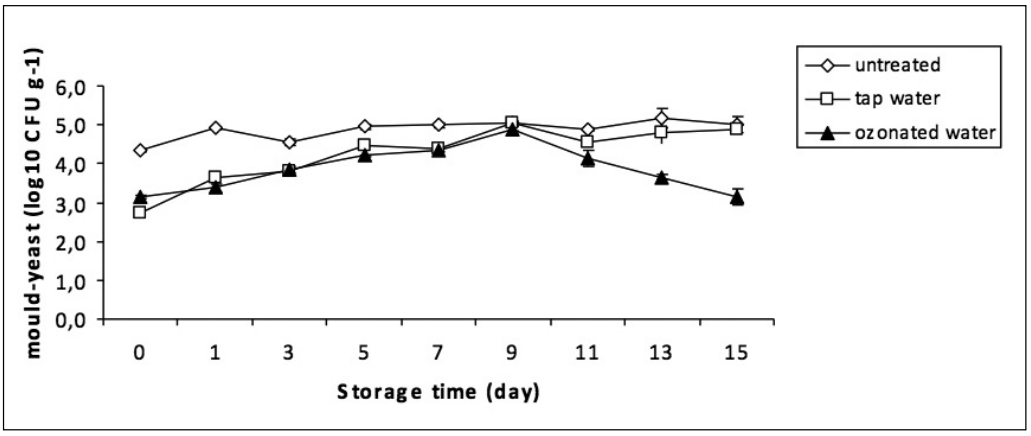


Fig. 5 - Changes in mould and yeast counts of untreated and treated carrots with tap water (5 min) and ozonated water (1.5 ppm/5 min) during storage period.
The bars represent standard deviations.

carrots treated with ozonated and tap water were found significantly different for the storage period of day 7 to day 15 (p<0.05). After 11 day of storage, TMAB counts of samples treated with ozonated water were sharply decreased and the initial bacterial counts were reduced by 1.12 log CFU/g at the end of storage period (Fig. 4). BELTRAN *et al.* (2005) reported that after 13 days storage of fresh - cut lettuce at 4°C, the microbial loads increased but the treatment reduced final microbial counts by 1.8 log unit when compared to control.

Washing waters significantly reduced mould and yeast counts of carrots (1.18 log CFU/g) at day 0 (p<0.05) (Fig. 5). Then the numbers were increased till the day of 9 (p<0.05) and come to the same level at day 9 (p>0.05) (Fig. 5). Significant effect of ozonated water on mould and yeast count of carrot was observed for the storage period of day 11 to day 15 (p<0.05). The results of the study showed that ozonated water effectively curtailed initial mould-yeast counts by 1.18 log CFU/g during storage (Fig. 5). In general, bacteria were more prone to ozone action in comparison to moulds and yeast (ALEXOPOULOS *et al.*, 2013). On the other hand, in this study the reduction obtained in TMAB count (1.79 log CFU/g) was found greater than the reduction in mould and yeast count at day 0. Similar finding was reported by CHAUHAN *et al.* (2011), who treated fresh - cut carrots with ozonated water (1:2 w/v; 200 mg $O_{3/}$h) for 10 min and observed reduction in standard plate count and mould - yeast count by 2.17 and 0.62 log cycle at day 0. The advantages of ozone treatment in the maintenance of sensory quality of carrot sticks during storage at low temperature were also detailed by CHAUHAN *et al.* (2011). Although possible effect in sensorial properties of treated carrots was not evaluated in this study, positive effects of ozonation were visually observed during storage period.

CONCLUSION

The findings of this study showed that ozonated water adds an additional reduction in natural background microflora of carrots. Ozonated water was significantly effective in reducing S. Typhimurium counts on carrots, depending on concentration and exposure times used. Moreover, ozonated water was found effective in maintaining the microbiological quality of carrots during storage period compared to untreated and tap water treated samples. Although limited reductions were achieved by ozone washing, the advantages, such as practical production by using ozone probes, wide antimicrobial spectrum and absence of by products make it strong alternative sanitizer for low contaminated fresh produce.

ACKNOWLEDGEMENTS

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PAPER

SWORDFISH STEAKS VACUUM-PACKED WITH *ROSMARINUS OFFICINALIS*

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ABSTRACT

In order to investigate the shelf life of swordfish steaks vacuum-packed with *Rosmarinus officinalis*, microbiological, chemical and sensorial analyses were carried out after 1, 3, 6, 8, 12 and 16 days of storage. In the treated samples, total volatile bases ranged from 7.35 to 13.05 mg/100 mL and trimethylamine from 1.21 and 7.9 mg/100 g. No significant change in the levels of ninhydrin-positive substances was observed. As regards the biogenic amines (BA), cadaverine increased linearly to 5.6 and 4.8 mg/100 g, and histamine to 4.9 mg/100g and 3.9 mg/100 g in the control and treated sample respectively. As regards the other BAs, no significant differences between the two samples were found. The effects of rosemary were reflected by a lower peroxide value of 2.72 mEq/kg in the treated steaks compared to 3.33 mEq/kg in the control sample at the end of the trial. The initial H_2S-producing bacteria and psychrotrophic counts of 3 and 5 $\log_{10}$ cfu/g increased to 5 and 6.2 $\log_{10}$cfu/g after 16 days of storage in the treated samples. *Salmonella*, *Staphylococcus aureus*, *Escherichia coli* and *Listeria monocytogenes* were not detected in any sample. The addition of rosemary in combination with vacuum packaging extended the shelf life of swordfish steaks by four days.

- Keywords: Rosmarinus, shelf life, swordfish, vacuum packaging -

INTRODUCTION

Swordfish is highly appreciated in Mediterranean countries and, like other large pelagic fish, it is usually sold in steaks or fillets. Demand for such forms of ready-to-cook and fresh/chilled seafood is constantly increasing (FAILLER *et al.*, 2008; PEPE *et al.*, 2007; PEPE *et al.*, 2010). Since many consumers today demand healthier foods, there is also increasing demand for milder, more natural ways of preserving food. Such a need could be met by the use of natural substances in combination with suitable packaging to prolong shelf life without adversely affecting product quality. This has concentrated attention on the wide range of effective, naturally-occurring, antimicrobial and anti-oxidant compounds which could be exploited as a means of preserving seafood. From this wide range of natural products, certain spices and aromatic herbs were investigated for their anti-oxidant and anti-microbial benefits in food preservation (SADOK *et al.*, 2004; GÓMEZ-GUILLÉN and MONTERO, 2007; FERNÁNDEZ *et al.*, 2009; TAJKARIMI *et al.*, 2010).

The aromatic herb *Rosmarinus officinalis*, commonly known as rosemary and used in Mediterranean countries to give special aromas or flavours to foods, is also known to have antioxidant and antimicrobial properties (BARATTA *et al.*, 1998; PÉREZ and MATEOS 2002; ALBU *et al.*, 2004). Its antioxidant properties have been attributed to the presence of various phenolic acids and flavonoids (SEWALT *et al.*, 2005), and interest in rosemary extract has increased since it was recognized as safe for use in food and was included in the GRAS list (EFSA 2008). Under European law (Directive 95/2/EC), extracts of rosemary may now be used as an additive in foodstuffs and E 392 has been assigned as its E number.

In this study, ground *Rosmarinus* came under consideration not only to satisfy consumer demands for more natural ingredients, but also as a means of giving a rustic and homemade appearance to the product.

Seafood is perishable and, due to the particularly rapid spoilage of the flesh, cutting or filleting fish further shortens its shelf life. The addition of natural preservatives such as herbs could extend the shelf life of fish fillets or steaks. *Rosmarinus* was used as an antibacterial agent in aquaculture (ABUTBUL *et al.*, 2004) and many studies have been carried out on seafood treated with rosemary extract involving frozen, cooked or salted fish (VARELTZIS *et al.*, 1997; SERDAROGLU and FELEKOGLU 2005; LUGASI *et al.*, 2007; ÖZOGULET *et al.*, 2009; ÖZYURT *et al.*, 2010). Some authors have studied the effects of *Rosmarinus* on freshly filleted, sliced and minced fish in refrigerated storage (AKHTAR *et al.*, 1998; DEL NOBILE 2009; KENAR *et al.*, 2010; UÇAK *et al.*, 2011).

The objective of this study was to investigate the effects of the addition of ground leaves of *Rosmarinus officinalis* on the shelf life of vacuum-packed swordfish steaks in refrigerated storage.

MATERIALS AND METHODS

Sampling

Fresh swordfish (*Xiphias gladius)* from the Tyrrhenian Sea (southern Italy) weighing 33 kg, was purchased on the day of capture, stored on ice and delivered to the laboratory 12 h after the docking of the fishing vessel. The fish was washed with tap water and drained. Steaks weighing 100 ± 0.1 g and 1cm in thickness were cut aseptically. The first batch of these samples was kept as a control; the second was aseptically spread with ground *Rosmarinus officinalis*, 0.02 g/ 100 g sample, obtained by freeze-drying the plant. The powder was pasteurized before use. Each batch comprised 12 samples which were then vacuum-packed in laminated film bags (HAFLIGER film type 145 bags; diffusion coefficient of 35 cm^3/24h m^2 bar to O_2 and 150 cm^3/24h m^2 bar to CO_2) and stored at 4°C. Steaks were sampled after 1, 3, 6, 8, 12 and 16 days of storage. Each evaluation was performed in duplicate.

Sensory analysis

Sensory evaluation of the vacuum-packed steaks was conducted by five trained panellists. Each sample was classified following the panel scheme of RUIZ-CAPILLAS and MORAL (2001). General appearance, colour, odour, flavour, water content, firmness, substantial, stiffness, and succulence were evaluated. Each characteristic was scored using a zero to ten attribute grading scale with 10 corresponding to the highest score for each parameter. Samples scoring less than 4 were rejected.

Microbiological analysis

In every sampling, an aliquot of 30 g, obtained by taking 10 g aseptically from three different sites of the steaks (the internal, middle and external parts), was homogenised in a stomacher for 60 seconds. Subsequently, only 10 g of homogenate were aseptically taken and decimally diluted in sterile maximum recovery diluent (CM 733 Oxoid). The total viable number of aerobic bacteria and psychrotrophic plate counts were determined as streak plate counts on marine agar after three days of incubation at 20°C and after 10 days of incubation at 7°C, respectively (Marine agar, BD Difco, Milan). *Enterobacteriaceae* counts were made using pour plates of 5 ml tryptone soya agar (TSA), and, after incubation at 20°-25°C for 2 h, the plates were overlaid with 12-15 mL of violet red bile glu-

cose agar (25°C, 48 h) (TSA/VRBGA, Merck). Hydrogen sulphide (H_2S)-producing bacteria were counted by pour plating with an overlay of Iron Agar Lyngby (25°C, 3 days) (IAL, Oxoid). Lactic acid bacteria (LAB) were enumerated using pour plates of MRS (de Man, Rogosa and Sharpe agar, Oxoid) after 5 days incubation at 30°C. Bacterial numbers were expressed as log CFU per gram of sample.

Analyses of total coliform and *E. coli* were conducted using the three-tube most probable number (MPN) methods (HARRIGAN and MCCANCE 1976; FDA, 1992). Lauryl sulphate tryptose broth (LST broth) and brilliant green lactose bile (2%) broth (BGLB broth) were used for presumptive and confirmed tests for total coliform, respectively. *E. coli* was determined by using the LST broth and EC broth. Cultures that showed positive production of gas were then confirmed by eosine methylene blue agar (EMBA) and IMViC tests.

Coagulase-positive *Staphylococcus aureus* was enumerated by plating on Baird-Parker agar (Oxoid) following the surface plate method. The incubation temperature used was 37°C (18–24 h) (ICMSF 1992). *Salmonella spp.* were detected in accordance with the FDA Bacteriological Analytical Manual (FDA, 1992) using a lactose broth enriched with selenite cysteine broth (SCB), tetrathionate broth (TTB) and Rappaport Vassiliadis broth (RVB) and plated on three selective plates, viz., Hektoen enteric agar (HEA), bismuth sulphite agar (BSA) and Xylose lysine desoxycholate agar (XLD) (ANDREWS and HAMMACK, 2003).

Listeria spp. was determined in accordance with the HITCHINS (2003) method using a preenrichment at 30° C for 4 h in buffered *Listeria* enrichment broth (BLEB). Incubation for selective enrichment continued at 30°C for a total of 48 h, adding Listeria Selective Enrichment Supplement SR141.

Chemical analysis

A single steak from each time point was minced using sterile scissors, and 10g were homogenised on ice with 5 ml ultrapure water (ELGA, UHQPS) for 1 min in a Polytron homogeniser (type PT 1200). A Mettler model PHM pH meter at room temperature was utilised for pH measurements. For the chemical analyses, 5 ml of 6% perchloric acid were added to the whole homogenised sample, and the extract was homogenised for a further 1 min. Homogenates were centrifuged at 14000 g for 20 min, and the supernatants were used for trimethylamine (TMA) (SADOK *et al.*, 1996 a), ninhydrin-positive substances (NPS) (SADOK *et al.*, 1996), total volatile bases (TVB-N) (RUIZ-CAPILLAS *et al.*, 1999) and nitrate/nitrite analyses (MONSER *et al.*, 2002). The peroxide value (PV), expressed in milliequivalents of peroxide oxygen per gram of fat, was determined by iodometric titration of the extracts after the addition of acetic acid (AOAC 1994). Contents of the biogenic amines (BA) putrescine (PUT), cadaverine (CAD), spermidine (SPD), spermine (SPR), and histamine (HIS) were determined by means of a chromatographic direct method based on cation-exchange separation of the amines and detection with integrated pulsed amperometry (ANASTASIO *et al.*, 2010). A 10-g aliquot of homogenised sample was extracted with 0.375 M perchloric acid and purified by liquid-liquid partition by using n-hexane. The aqueous phase was filtered through a 0.2- mm-pore-size filter and injected for analysis. Separations were performed on an IonPac CS10 cation-exchange column coupled to an IonPac CG10 guard column (Dionex Co., Sunnyvale, CA) packed with 8.5 mm of solvent-compatible ethylvinylbenzene-divinylbenzene 55% XL substrate, agglomerated with 175 nm of sulfonated cation-exchange latex for a cation-exchange capacity of approximately 80 mEq per column. Isocratic separation was performed with an eluent containing 1 M sodium perchlorate– 0.375 M perchloric acid–water (81:5:14 [vol/vol/vol]) at a flow rate of 1 ml/min. Chromatographic separation was achieved with a model 45001 liquid chromatograph (Dionex Co.) and an ED40 detector with an electrochemical cell equipped with an Au working electrode and a reference electrode in Na form (pH-Ag-AgCl; Dionex Co.).

Statistical analysis

Where appropriate, normally-distributed data ($P > 0.05$, K-S Lillifors test) on TMA, TVB, TMAO, NPS and biogenic amine concentrations were subjected to analysis of variance (one-way ANOVA) using SPSS (sub-programme of the Statistical Package for the Social Sciences) on a PC. The level of significance for the F-test, used in conjunction with ANOVA variance data, was at the 95% level of confidence. Leverage correction of all the data was applied. The variables were weighted with the inverse of the standard deviation of all the objects in order to compensate for the different scales of the variables.

RESULTS AND CONCLUSIONS

The changes which occurred during the storage periods in the control samples and in rosemary-treated samples are shown in Table 1. It can be observed that *Rosmarinus*-treated steaks still received a very high score for colour and flavour even after eight days of storage. Treated samples were regarded as acceptable until day 12 when there was some discoloration although they still had a pleasant flavour and showed good firmness and absence of liquid in the package. Untreated samples, except for colour and firmness of kneading, were considered unacceptable

Table 1 - Sensory evaluation in control (CF) and treated fillets (TF) during 16 days of storage.

Parameters		Day of storage											
		1	std	3	std	6	std	8	std	12	std	16	std
Appearance general	CF	9,8	±0,3	9	±0,90	8	±0,0	7	±0,0	4,1	±0,29	3,5	±0,0
	TF	9,8	±0,3	9,1	±0,89	8,4	±0,3	8	±0,0	5,5	±0,50	4	±0,0
Color	CF	9,9	±0,2	8,2	±0,05	7,5	±0,0	6,3	±0,25	4,2	±0,40	3,4	±0,21
	TF	9,9	±0,2	8,5	±0,25	7,9	±0,2	7,5	±0,1	5,4	±0,45	3,9	±0,1
Odor	CF	9,7	±0,5	8	±0,0	6,8	±0,2	5,2	±0,2	3,9	±0,11	3,2	±0,35
	TF	9,7	±0,5	8	±0,0	7	±0,0	6,4	±0,06	4,9	±0,2	3,9	±0,1
Flavor	CF	10	±0,0	8,4	±0,15	7	±0,0	5,4	±0,1	4,1	±0,11	3,4	±0,47
	TF	10	±0,0	8,4	±0,21	7,2	±0,2	6,6	±0,21	5,7	±0,43	4,1	±0,11
Watery	CF	9,8	±0,3	7,7	±0,29	6,1	±0,3	5,5	±0,0	4	±0,0	3,6	±0,0
	TF	9,8	±0,3	7,9	±0,11	6,5	±0,0	6	±0,0	5,2	±0,29	4	±0,0
Firmness	CF	9,9	±0,2	8,1	±0,11	6,5	±0,0	5,7	±0,31	3,8	0,29	3,2	±0,05
	TF	9,9	±0,2	8,1	±0,11	6,8	±0,2	6,2	±0,2	5,2	±0,86	3,8	±0,1
Substantial	CF	9,8	±0,3	7,8	±0,11	6	±0,0	5,2	0,25	3,9	0,11	3,3	±0,1
	TF	9,8	±0,3	7,8	±0,11	6,4	±0,1	5,9	±0,1	4,3	±0,0	3,9	±0,1
Stiffness	CF	9,6	±0,4	8	±0	6,5	±0,0	5,6	0,66	3,9	0,11	3,3	±0,1
	TF	9,6	±0,4	8	±0,0	6,8	±0,2	6,1	±0,2	4,4	±0,32	3,9	±0,1
Succulent	CF	9,7	±0,5	8,1	±0,11	6,1	±0,2	5,9	0,12	3,9	0,11	3,4	±0,4
	TF	9,7	±0,5	8,1	±0,11	6,7	±0,2	6	±0,0	4,3	±0,0	3,9	±0,1
Acceptability	CF	9,9	±0,2	8,1	±0,11	7,4	±0,2	5,4	0,21	3,9	0,11	3,4	±0,47
	TF	9,9	±0,2	8,3	±0,15	7,7	±0,1	5,9	±0,1	5,1	±0,11	3,8	±0,1

from day 8 because of a gradual loss of pearly lustre, a slight flavour of ammonia and the presence of liquid in the bag.

The flesh pH values increased progressively from 6.65 to 7.21 and from 6.60 to 7.01 after 16 days of storage in the control and the treated batches, respectively (data not shown). As shown in Table 2, aerobic bacteria counts were 6 log CFU/g until 12 days of storage in treated samples, while in the control steaks, the aerobic population reached 6.4 log CFU/g already after eight days of storage. The psychrotrophic bacte-

Table 2 - Microbiological Counts (Log10 Colony-Forming Units per Gram) during storage of treated fillets (TF) and control fillets (CF)

Storage day		1		3		6		8		12		16	
			dv		dv		dv		dv		dv		dv
Aerobic bacteria	CF	5,0	±0,01	5,2	±0,01	5,6	±0,01	6,4	±0,01	7,1	±0,01	7,5	±0,02
	TF	5,0	±0,02	5,1	±0,01	5,2	±0,02	5,3	±0,02	6,0	±0,01	6,7	±0,02
Psychotropic bacteria	CF	5,0	±0,01	5,2	±0,01	5,5	±0,01	6,0	±0,02	7,1	±0,01	8,0	±0,02
	TF	5,0	±0,01	5,2	±0,02	5,2	±0,03	5,3	±0,03	6,0	±0,02	6,2	±0,03
(H2S-producing bacteria	CF	3,1	±0,01	3,5	±0,02	4,5	±0,04	5,1	±0,02	6,7	±0,02	7,0	±0,02
	TF	3,1	±0,03	3,2	±0,03	3,4	±0,02	3,5	±0,02	4,5	±0,02	5,0	±0,03
Enterobacteriaceae	CF	2,5	±0,05	3,4	±0,04	3,8	±0,03	4,2	±0,02	4,5	±0,01	6,8	±0,03
	TF	2,5	±0,06	2,8	±0,05	3,0	±0,01	3,1	±0,02	3,2	±0,01	3,5	±0,04
LAB	CF	2,8	±0,03	4,3	±0,05	5,5	±0,01	6,3	±0,01	7,1	±0,02	7,5	±0,04
	TF	3,0	±0,02	3,8	±0,07	4,0	±0,01	4,5	±0,01	5,6	±0,01	6,8	±0,02

ria count at the beginning of storage was $\log_{10}$ 5 CFU/g in both groups (Table 2). In the treated steaks, a slight increase in the psychotropic population was observed, recovering a cell load of 5.3 log CFU/g against 6 log CFU/g in control samples, after eight days of storage. Microbial growth of H_2S-producing bacteria in the treated samples was delayed by rosemary treatment and counts remained low up to day 8. In the control steaks, these bacteria gradually increased and reached levels of $\log_{10}$ 5 CFU/g at the same sampling time. This different trend explains the higher degree of spoilage observed in untreated samples. The *Enterobacteriaceae* and LAB counts, starting after eight days of storage, were higher in the control samples compared to the treated ones. *Staphylococcus aureus, L. monocytogenes, E. coli* and *Salmonella* spp. were not detected in any of the samples throughout the entire storage period.

Changes in TVB-N levels, the most frequently used parameter to monitor seafood quality and freshness (GRAM and DALGAARD 2002), are depicted in Fig. 1. After three days of storage, significant differences ($p< 0.05$) were observed with lower TVB-N values in the *Rosmarinus*-treated steaks than in the control samples. Levels increased from an initial value of 7.35 mg/100 g flesh to a maximum of 13.05 and 20.96 mg/100g flesh in rosemary-treated and control fish, respectively. For up to 16 days of refrigerated storage, the swordfish TVB-N flesh levels were below the limit (30 mg/100g) above which fishery products are considered unfit for human consumption (CONNELL, 1995).

The initial low TMA content (1.21 ± 0.022 mg TMA/100g) was indicative of fish of good quality (Castell *et al.*, 1958). During refrigerated storage, as shown in Fig. 2, TMA increased exponentially in both the control and treated samples (R^2 = 0.99), with significantly lower TMA content ($P < 0.05$) in rosemary-treated samples. This low production was confirmed by low pH levels in the treated samples. In this sample, a significant increase in TMA levels occurred only after eight days of refrigerated storage.

TMA results from the reduction of TMAO by bacterial activity and, to a certain extent, from intrinsic enzymes. It is considered a valuable tool in evaluating the quality of refrigerated fish (VILLAREAL and POZO 1990).

Free amino acids in fish, often measured as ninhydrin-positive substances (NPS), reflect microbial spoilage and are precursors of BA, factors concerning food safety and indicators of fish decomposition (ANTOINE *et al.*, 2001). No significant NPS change occurred in any of the swordfish samples for up to 10 days of refrigerated storage, and a significant NPS increase subsequently occurred only in the non-treated vacuum-packed swordfish steaks (Fig. 3). This increase was probably due to the inhibitory effect of rosemary on a microbial component linked to NPS formation (DEL CAMPO *et al.*, 2000).

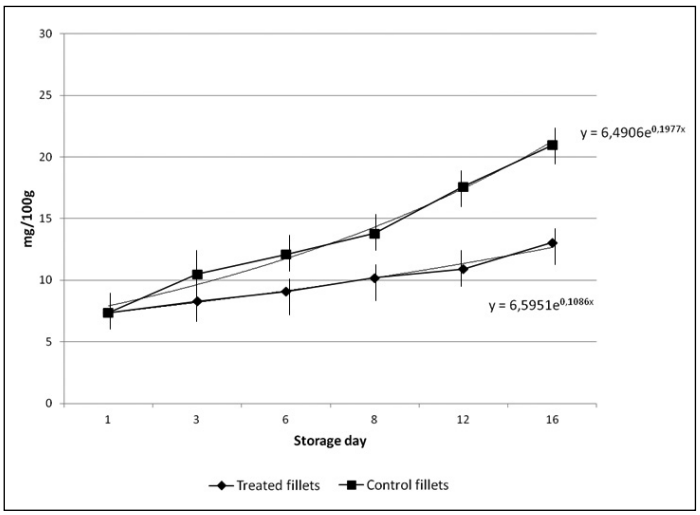


Fig. 1 - Change in total volatile bases-nitrogen (TVB-N) levels in control and in Rosmarinus-treated steaks samples during refrigerated storage. Vertical bar = ± SE (n = 6 each case).

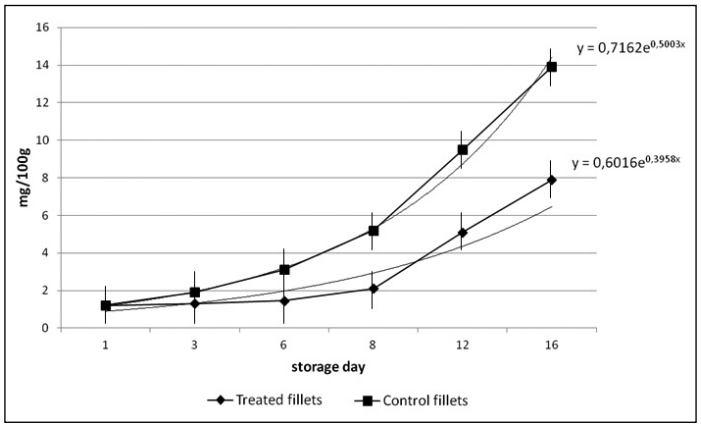


Fig. 2 - Change in trimethylamine (TMA) content levels in control and in Rosmarinus-treated steaks samples during refrigerated storage. Vertical bar = ± SE (n = 6 each case).

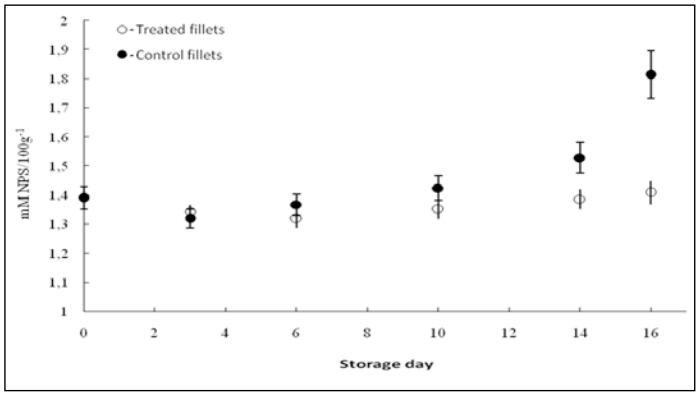


Fig. 3 - Change in total free amino acids levels measured as NPS in control and in Rosmarinus-treated steaks samples during refrigerated storage. Vertical bar = ± SE (n = 6 each case).

Regulations set maximum limits for nitrate in food such as fish at 146 mg /100g (HIRONDEL and HIRONDEL 2001). The initial level found in this study was far below this limit (0.75 ± 0.02

Table 3 - Biogenic amines profile (mg/100g) during storage of treated fillets (TF) and control fillets (CF).

Storage day		1	3	6	8	12	16
cadaverine	CF	1,12	2,1	2,9	3,5	4,5	5,6
	TF	1,12	2,1	2,6	3	3,6	4,8
putrescine	CF	0,035	0,3	0,8	1,7	2,1	2,9
	TF	0,035	0,4	0,7	1,6	2,3	2,8
histamine	CF	0,56	0,7	1,7	2,1	3,8	4,9
	TF	0,56	0,6	1,5	2	2,8	3,9
spermidine	CF	0,035	0,4	0,9	1,8	2,2	3,1
	TF	0,035	0,4	0,7	1,4	1,9	2,8
spermine	CF	nd	0,1	0,5	0,9	1,2	1,6
	TF	nd	0,1	0,4	0,9	1	1,6

mg/100g). Unexpectedly, the nitrate content showed a sharp decrease in all vacuum-packed swordfish steaks after only two days of storage (data not shown).

The massive nitrate reduction (LYHS *et al.*, 1998) could be related to its degradation to ammonia or to the formation of nitrosamines by specific bacterial action under vacuum packaging conditions. LYHS *et al.* (2001) signalled that the occurrence of *P. aeruginosa* and staphylococci in nitrate-containing fish samples, stored at 8° C, could result in product safety complications. Such an assumption requires, however, a thorough investigation given the hazard of nitrosamines in food.

BA are nitrogenous compounds commonly found in food and seafood, and the determination of their nature and quantities in the diet is of prime interest as they are involved in a multitude of basic metabolic processes (BARDÓCZ, 1995). Among the measured polyamines, in the beginning of the trial, CAD showed the highest level (1.12 mg/100g flesh), followed by HIS, and relatively low and similar levels of PUT and SPD (0.035 mg/100g). SPM was initially undetectable. After three days of refrigerated storage, both swordfish samples exhibited a steady increase in all BA (Table 3) with a significant regression coefficient (> 0.9 in most cases). Differently from BUGUEÑO *et al.* (2003) who suggested PUT and CAD as spoilage indicators in vacuum-packed cold smoked salmon, in this study only CAD seemed to play this role, since PUT reached levels < 3 mg/100g at the end of the experimental period in both groups.

Although concentrations of histamine (HIS) also increased during storage, they did not exceed the limit of 50 ppm established by the FDA or 100-200 ppm by EU Regulations for various fish species. Our data showed that the products analysed, even if consumed on the 16th day of storage, were unlikely to result in scombroid poisoning in humans. However, a significant difference between control and *Rosmarinus*-treated steaks was observed towards the end of storage with significantly lower levels of HIS in the latter. This could be explained by several factors; increasing pH could lead to a reduction in the decarboxylase-positive lactic flora activity and to lower amine production. A similar trend was observed in a previous study (MERCOGLIANO *et al.*, 2013) in tuna-based products. Furthermore, low levels in treated samples could be attributed to the unfavourable conditions for the growth of *Enterobacteriaceae* and LAB which can accumulate a high amount of HIS, as reported by BRINK *et al.* (1990) for dry fermented sausages. In our study, this result was confirmed by the low microbial counts of the above-mentioned microbial groups detected in treated samples, as previously described. Peroxidation of lipids in fresh and processed seafood is one of the main causes of product deterioration: it induces off-flavour formation which negatively affects quality (COUPLAND and MCCLEMENTS, 1996). The use of rosemary as an antioxidant for food has been proposed in several reports (FRUTOS and HERNÁNDEZ-HERRERO, 2005; ESTÉVEZ *et al.*, 2007).

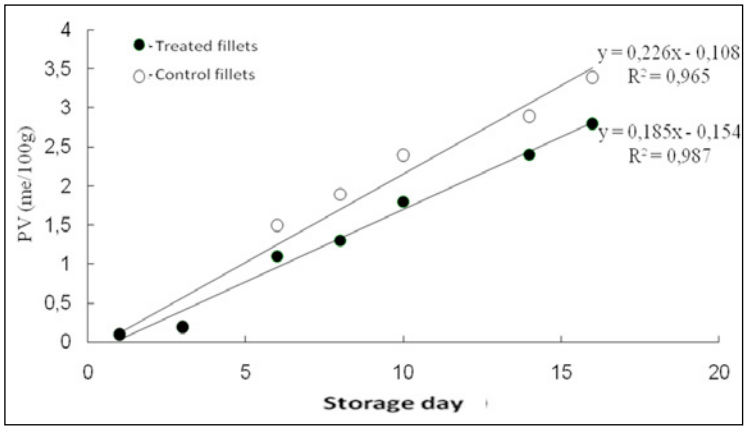


Fig. 4 - Change in the peroxide values in control and in Rosmarinus-treated steaks samples during refrigerated storage. Vertical bar = ± SE (n = 6 each case).

In this study, lipid oxidation was evaluated by monitoring changes in the peroxide values in swordfish muscle during 16 days of storage. Figure 4 shows that PV changes in control and rosemary-treated swordfish samples followed a similar pattern of changes, increasing ($R^2 > 0.9$ in both group) linearly with the duration of storage but with significantly lower PV values in the treated group.

In conclusion, our findings show that treatment with *Rosmarinus* extends the shelf life of vacuum-packed refrigerated swordfish steaks. Sensory analyses, combined with chemical and microbiological data, demonstrate that safe storage of samples can be extended as much as four days by adding ground rosemary.

Since naturalness has become one of the catchwords for food attractiveness, application of ground *Rosmarinus* on swordfish steaks may not only be cost-effective to extend shelf life but also has potential for being perceived as a natural, traditional processing method, hence making the product more highly appreciated by consumers.

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PAPER

SOME PHYSICOCHEMICAL CHARACTERISTICS OF RAW MILK OF ANATOLIAN BUFFALOES

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ABSTRACT

This research was carried out to determine some constituents and properties of raw milk samples of Anatolian buffaloes. Raw milk samples were collected from the different areas of the Tokat province of Turkey and analyzed for density, acidity, urea, free fatty acids (FFA), citric acid, freezing point (FPD), and pH. Milk samples were collected in February, March, and April 2012. Results of the research showed that the averages of raw milk density, acidity, urea, free fatty acids, citric acid, freezing point degree, and pH were determined as 1029.66 g/cm^3, 8.26 ^{o}SH, 0.047%, 4.78 mmol/10L, 0.13%, -0.56 ^{0}C, and 6.56, respectively. As a result, the effects of lactation number, calving age, village, herd, sampling time, and stage of lactation on the determined parameters were found to be statistically significant (P<0.01).

- Keywords: buffalo milk, density, acidity, urea, free fatty acids, citric acids, freezing point, pH -

INTRODUCTION

Milk is universally recognized as a complete diet owing to its essential components for human nutrition. Therefore, it is considered as one of the most important foods for human beings (SHARİF and MUHAMMED, 2009). Milk quality is as important as the quantity of milk production to the dairy industry. The physical properties and chemical composition of buffalo milk vary according to the animal genotype, and are influenced by several factors such as the lactation stage, parity, calving age, and season. Buffaloes are used more frequently as a draft animal in rural places in Turkey. Also, buffalo milk, as one of the most valuable products, is among the main protein sources for poor or rural breeders and provides a significant income for the rural economy (BORGHESE, 2005; YILMAZ *et al.*, 2011). Buffalo breeding provides 12% of total milk production in the world (AHMAD *et al.*, 2008). The Anatolian Buffalo is the second most important dairy species in Turkey. In recent years, while buffalo population has increased throughout the world, the buffalo population in Turkey has begun to decline (SAHİN *et al.*, 2011). The buffaloes in Turkey are called Anatolian buffalo and they are among the Mediterranean Buffaloes, which are a subgroup of river buffaloes (SOYSAL *et al.*, 2005). The recorded number of Anatolian buffaloes in Turkey was 366,150 in 1991 and decreased to 107,435 in 2012 (ANONYMOUS, 2012). They are mostly bred in North, Middle, West, East, and Southeast Anatolia in Turkey (ATASEVER and ERDEM, 2008). Anatolian buffaloes are particularly bred for milk production and they are slaughtered for meat production after their productive years in Turkey (SEKERDEN, 2001). Anatolian buffaloes are a considerably preferred breed in the different regions of Turkey due to their resistance to diseases and lower feed consumption. Notwithstanding, the genetic structure of buffaloes is principally taken into consideration and the importance of environmental factors remains secondary to many dairy operations in Turkey. The scientific literature concerning the description of the density, acidity, urea, free fatty acids, citric acid, freezing point, and pH, and understanding the effects on these physical components of buffalo milk is limited. Thus, there is limited research on the density, acidity, free fatty acids, citric acids, freezing point, and pH in raw buffalo milk in Turkey.

The aim of this study was to define the density, acidity, urea, free fatty acids (FFA), citric acid, freezing point degree (FPD), and pH, and to identify and quantify environmental factors affecting some milk chemical compositions in Anatolian buffaloes.

MATERIALS AND METHODS

Location of the experiment

This study was carried out in the Tokat province in the mid-Black Sea Region of Turkey. Located between 35° 27' and 37° 39' East longitudes, and 39° 52' and 40° 55' North latitudes. The district has a climate with a transition feature between the Black Sea Maritime climate and the Anatolian Continental climate. The long-term average yearly temperature ranges from 8.1 to 14.2°C. Average relative humidity is between 56 and 73% (MARA, 2011).

Sample collecting

Anatolian buffaloes, raised in different villages of Tokat, were examined between February to April 2012. More than 636 samples were collected. Lactating buffaloes were grouped into three lactation stages (1^{st}, 2^{nd}, 3^{rd} month (1: early); 4^{th}, 5^{th}, 6^{th} month (2: mid); and 7^{th}, 8^{th} and 9^{th} month (3: late) and a total of seven parity groups (1- ≥7 parities). Sampling times were evaluated in three subgroups (February, March, and April). Buffaloes are typically milked once in the morning before being moved to pasture. Therefore, raw milk samples (about 50 mL) were obtained from each animal during the morning milking in plastic sterile bottles containing (one tablet) of 2-bromium-2-nitroprophane-1,3 diol (Bronopol) and kept cold until analyzed.

Methods of analysis

FOSS Milko Scan TM 120 (calibrated with appropriate buffalo standard, Foss electric, Denmark) was used to determine density, acidity, free fatty acids, citric acid, and freezing point in raw milk samples. It is founded on well-identified IR-technology utilized in other FOSS Milko Scans, and compatible with IDF (International Dairy Federation) principles and AOAC (Association of Official Analytical Chemists) formal procedures.

The pH was measured using a digital pH-meter (HI 8314, Hanna Instruments, Italy), standardized with pH 4 and 7 buffers.

Statistical analysis

In the study, stage of lactation, parity, farm, and season were evaluated as independent variables. All statistical analyses were conducted using the SPSS statistical package program (SPSS 17.1). The data were examined by analysis of variance (ANOVA).

The model was as follows:

$$Y_{ijklmn} = \mu + a_i + b_j + c_k + d_l + f_m + e_{ijklmn}$$

Table 1 - Descriptive statistics of some physicochemical characteristics of Anatolian buffaloes milk.

	N	Mean	SE	Minimum	Maximum
Density (g/cm³)	636	1029.66	0.306	1028	1033
Urea (%)	609	0.047	0.001	0.036	0.057
Acidity (°SH)	636	8.26	0.153	5.96	9.94
Free fatty acids (mmol/10 L)	304	4.78	0.375	3.22	6.35
Citric Acid (%)	636	0.13	0.002	0.11	0.15
Freezing Point Degree (°C)	636	-0.56	0.007	-0.46	-0.66
pH	328	6.56	0.008	6.01	7.00

Where:

Y_{ijklmn}: Observation value for various physicochemical characteristics
μ: Population mean
a_i: Effect of the parity (k: 1, 2, ...7)
b_j: Effect of villages (j: 1, 2,....12)
c_k: Effect of the calving ages (I =3, 4, 5, ...9)
d_l: Effect of sampling time (February, March, April)
f_m: Effect of the stage of lactation (Early, Mid, Late)
e_{ijklmn}: Random residual effect

RESULTS AND DISCUSSION

The means of density, acidity, urea, free fatty acids, citric acids, freezing point degree, and pH values were determined to be 1029.66 g/cm³, 8.26 °SH, 0.047%, 4.78 mmol/10L, 0.13%, -0.56 °C, and 6.56, respectively. Descriptive statistics of the variables studied in this study are presented in Table 1.

The results obtained from the preliminary analysis of the means of various chemical characteristics for lactation number, village, calving age, sampling time, and stage of lactation are presented in Figs. 1, 2, 3, 4, and 5, respectively. The density and pH of all the raw milk samples were found to be 1029.66±0.306 g/cm³ and 6.56±0.008, respectively. Small variations were found for the two parameters in all the milk samples. The density is mainly due to the water content present in the sample, and pH is the parameter that determines the sample alkalinity and acidity. Furthermore, density is a measure that provides information about the purity of the raw milk. The pH range found in the current study was similar to the findings in previous investigations (6.38±0.60 to 6.77±0.88; 6.59±0.59 to 6.93±0.57; 6.58 to 6.95; 6.62 to 6.64; 6.45 to 6.61) (REHMAN and SALARIA, 2005; IMRAN *et al.*, 2008; BRAUN and PREUSS, 2008; SAMEEN *et al.*, 2010; YANG *et al.*, 2013). Furthermore, this pH value (6.65) was lower than those reported by GHAFOOR *et al.* 1985, Han *et al.* (2007), BRAUN and PREUSS (2008), and ENB *et al.* (2009). Additionally, MÉNARD *et al.* (2010) reported that buffalo milk pH was 6.74. The current research produced results that support the findings of a great deal of the previous work in this field. The density and pH of buffalo milk were reported to be 1033 g/cm³ and 6.75, respectively, by MAHMOOD and USMAN (2010). KANWAL *et al.* (2004) stated that buffalo milk pH, acidity, and densi-

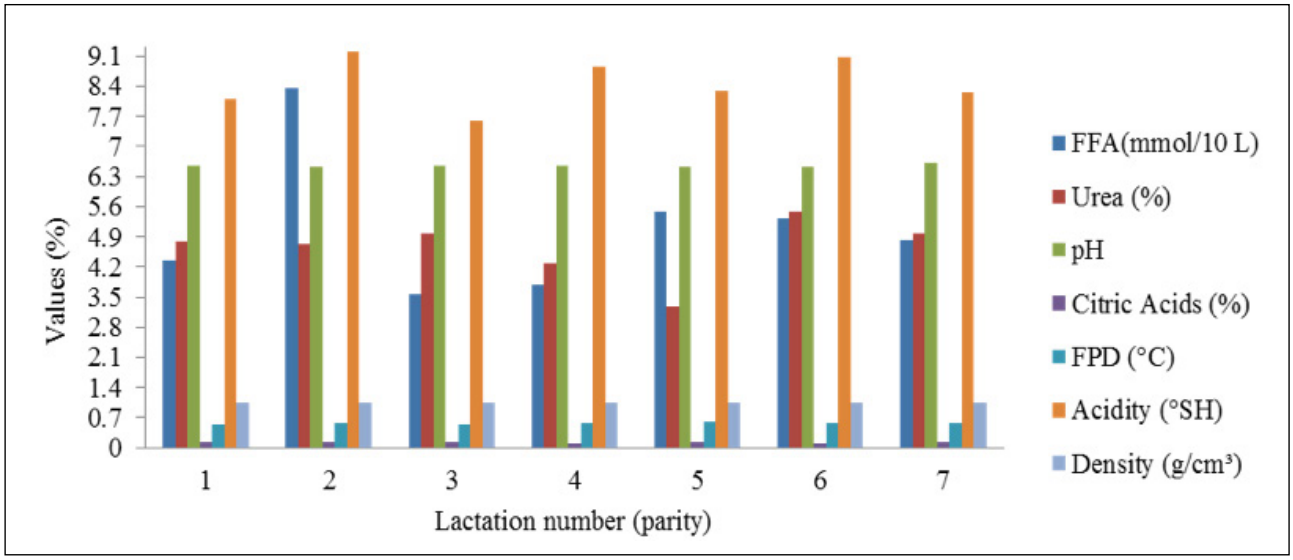


Fig. 1 - Chemical composition of buffalo milk according to parity.

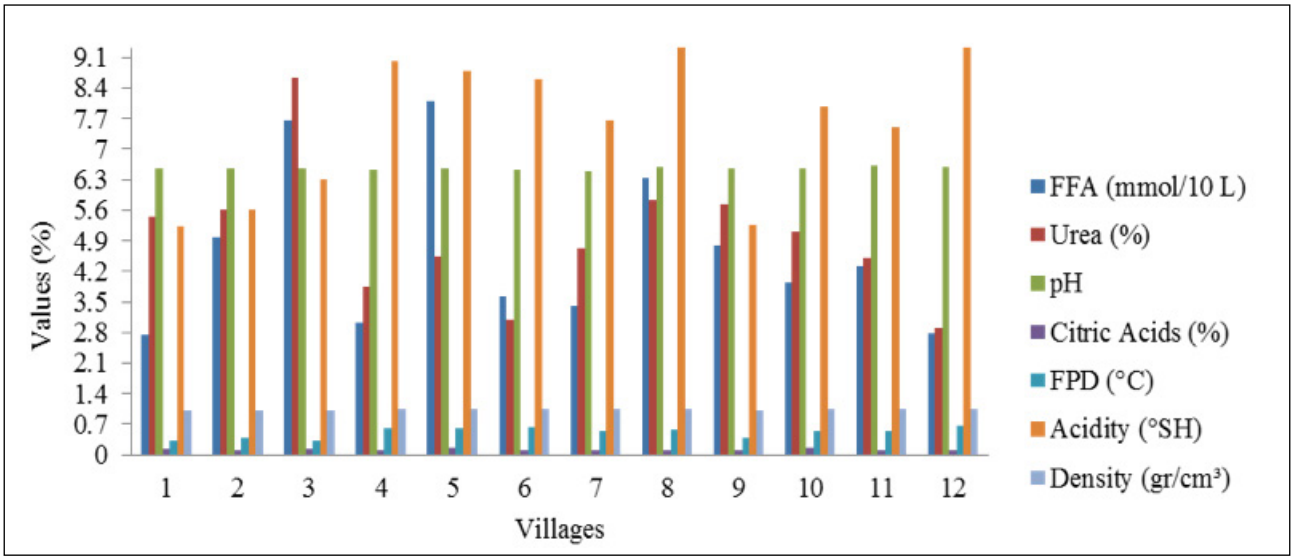


Fig. 2 - Chemical composition of buffalo milk according to villages.

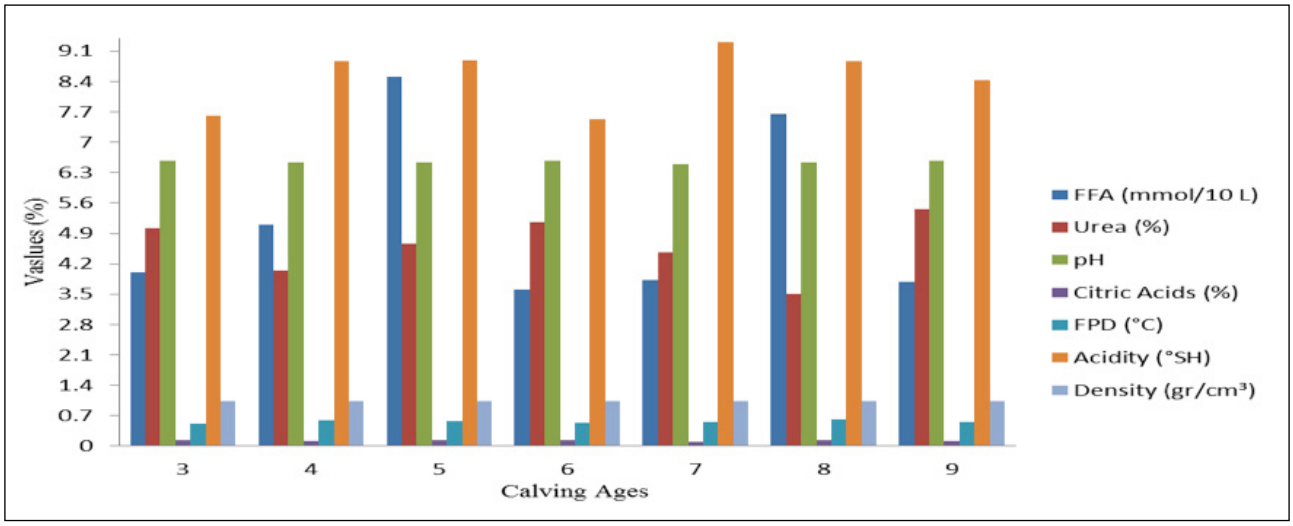


Fig. 3 - Chemical composition of buffalo milk according to calving ages.

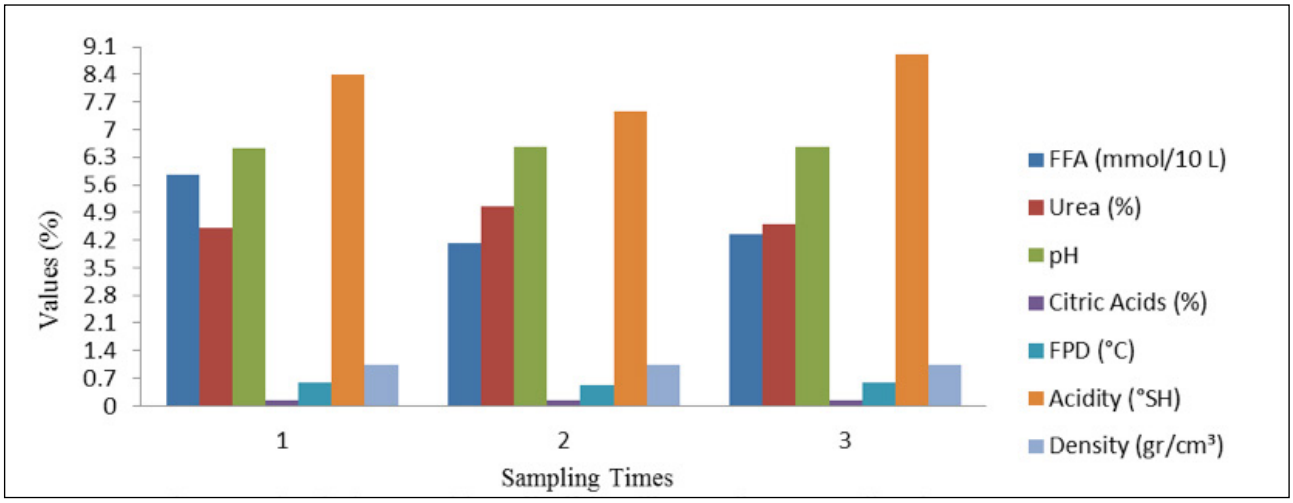


Fig. 4 - Chemical composition of buffalo milk according to sampling times.

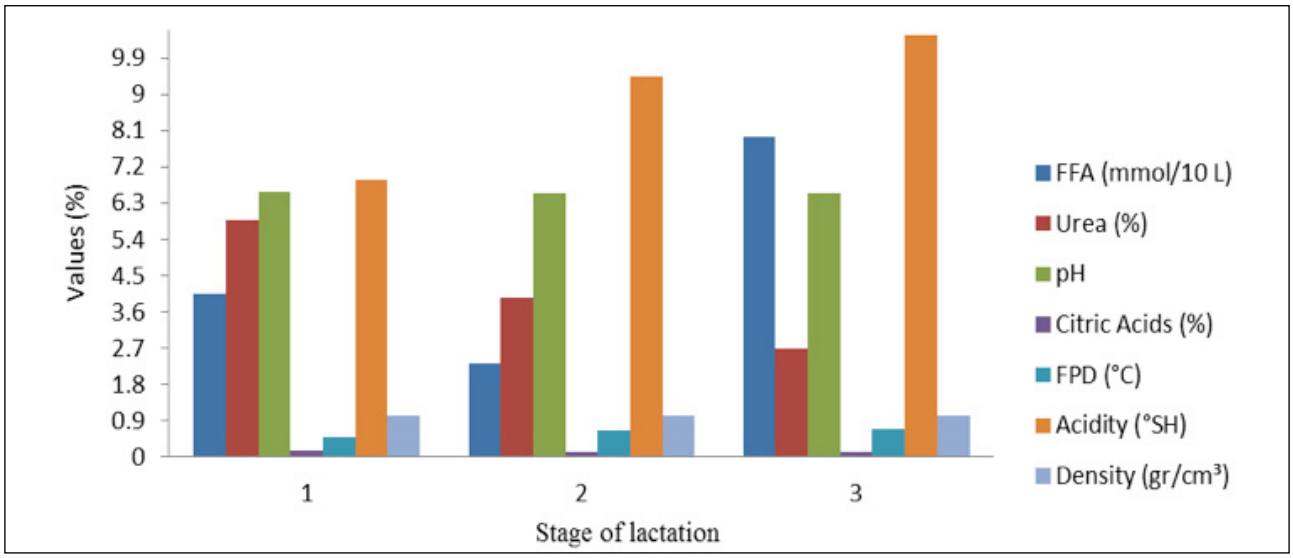


Fig. 5 - Chemical composition of buffalo milk according to stage of lactation.

ty were 6.64, 0.133, and 1020 g/cm^3, respectively. HAQUE *et al.* (2012) declared that buffalo milk pH was 6.70. Furthermore, AHMAD *et al.* (2005) reported that buffalo milk pH and density were 6.58 and 1032 gr/cm^3, respectively. The density value was lower than the findings of some previous research results (PADGHAN *et al.*, 2008; BRAUN and PREUSS, 2008). HAN *et al.* (2007) declared that buffalo milk pH was 6.53 for Murrah breed, and 6.39 for Nili Rawi breed. Buffalo milk densities were 1034, 1032, 1032, and 1033 gr/cm^3, for winter, spring, summer, and autumn seasons, respectively (AURELIA *et al.*, 2009). The mean pH (6.56 ± 0.008) of Anatolian buffalo milk was similar to the values reported by HAN *et al.* (2007), SEKERDEN and AVSAR (2008), PADGHAN *et al.* (2008), and GÜRLER *et al.* (2013), but higher than those of AURELIA *et al.* (2009) and COROIAN *et al.* (2013). Turkish Food Regulations report that the density of raw buffalo milk is 1028 g/cm^3 (ANONYMOUS, 2000). The present results are similar to this standard. This value (1029±0.306 g/cm^3) is lower than findings of FRANCISCIS *et al.* (1988) and similar to the results of ZAMAN *et al.* (2007). In addition, Ahmad *et al.* (2008) reported that buffalo milk pH was 6.81. The results of the present research are consistent with those of KHAN *et al.* (2007), who found that the density and pH were 1032 g/cm^3 and 6.37 for swamp buffaloes, respectively, and 1032 g/cm^3 and 6.57, respectively, for water buffaloes. The average pH of milk samples collected from swamp and water buffalo were within the normal range.

In this study, the mean value of the milk urea content was 0.047%. This result similar to AYASAN *et al.* (2011), who reported that milk urea content was 0.04% for Holstein cattle in Turkey. Milk urea content was determined to be 3.78 mg/100 mL of Anatolian buffaloes by SEKERDEN and AVSAR (2008), who claimed that milk urea content only affected feeding levels. In addition, the level of the feeding regime has also been reported to have an effect on milk urea content by ABREU (2008).

In this study, the mean value of the milk urea content was 0.047%. This result similar to AYASAN *et al.* (2011), who reported that milk urea content was 0.04% for Holstein cattle in Turkey. Milk urea content was determined to be 3.78 mg/100 mL of Anatolian buffaloes by SEKERDEN and AVSAR (2008), who claimed that milk urea content only affected feeding levels. In addition, the level of the feeding regime has also been reported to have an effect on milk urea content by ABREU (2008).

The protein/energy ratio of animal feed had an effect on milk urea concentration (BAKER *et al.*, 1995; AYASAN, 2009). Milk urea levels may change depending on a number of factors.

Milk composition, breed, season, time of feeding, somatic cell count, feeding regime, feeding method, and water and dry matter consumption are among the most important of these factors (NOUROZI *et al.*, 2010; ROY *et al.*, 2011). These findings further support the results of the study of ROY *et al.* (2005), who reported that feeding regimes had a significant effect on raw milk urea concentration.

Furthermore, the same researchers revealed that this effect might be due to the difference in the quality and type of protein between the diets and the feeding strategy of the research. The composition of milk free fatty acids is dependent on various factors, such as stage of lactation, genetic variation, breed, calving age, animal health, and feed composition (GARNSWORTHY *et al.*, 2006; QURESHI *et al.*, 2010).

In the present study, milk free fatty acid content was found to be 4.78 mmol/10L. Similar results were obtained by some researchers (HOFI *et al.*, 1977; BERI *et al.*, 1984; TALPUR *et*

al., 2007). On the other hand similar results were obtained for Holstein cattle by FILIK *et al.* (2011) and AYAŞAN *et al.*, (2012). Sharma *et al.* (2000) reported that milk fatty acid content was 0.58±0.01, 0.65±0.02, and 0.84±0.07 according to buffalo during lactation stages (early, mid, and late stages, respectively).

The freezing point of raw milk is an important feature to determine the amount of water added (AYDIN *et al.*, 2010). In this experiment, the average freezing point was determined as -0.56°C in milk samples. Similarly ROSENMAN and GARRY (2010) reported that the buffalo milk freezing point was -0.52°C. The freezing point of buffalo milk in Germany ranged from -0.55° to -0.51°C (BRAUN and PREUSS, 2008); FILIK *et al.* (2011) and AYAŞAN *et al.* (2012) reported that the freezing point of Holstein cattle milk is -0.51° and -0.52°C.

In this study, milk citric acid content was determined to be 0.13%. According to FILIK *et al.* (2011), the milk citric acid content of Turkish Holstein cattle was found as 0.11%. This value is in agreement with findings of AYAŞAN *et al.* (2012) for Holstein cattle in Turkey.

The degree of acidity is a good indicator of whether or not it was held under appropriate conditions from the time of milking until it is processed (UNAL and BESLER, 2006). The mean acidity percentage of the buffalo milk was 8.26±0.153 °SH. It has been explained in the Turkish Food Regulations that the acidity of raw buffalo milk is not higher than average 8 °SH (0.14-0.22 %). It can be seen from Table 1 that this is similar to the normal value. The values of the acidity in buffalo milk were in accordance with the findings REHMAN and SALARIA (2005), PADGHAN *et al.* (2008), and COROIAN *et al.* (2013). This value is similar the study of BOVERA *et al.* (2002), who determined that buffalo milk acidity ranged from 8.37 to 8.81 °SH. It was reported by SEKERDEN and AVSAR (2008) that the acidity percentage of buffalo milk was 0.17%. These results are in agreement with HAQUE *et al.* (2012), who reported that buffalo milk acidity was 0.21. EL AGAMY *et al.* (1998) found that the mean value of acidity for buffalo milk was 0.18. It is clear that the pH values had an opposing trend from acidity percentages. The results of the present study are in agreement with those of KHAN *et al.* (2007), who found that the acidity percentage was 0.16% for swamp buffaloes, 0.15% for water buffaloes, and 0.16% for the overall mean. The results of the present investigation are in agreement with the findings of various researchers (ENB *et al.*, 2009; SAMEEN *et al.*, 2010). Acidity values found in buffalo milk were lower than the findings of Mahmoud and Usman (2010). The first acidity in milk is due to the amount of casein phosphate, citrate, and carbon dioxide. However, later, the bacterial activity increases and lactic acid is formed, and thus the acidity of the milk increases. Extra acidity in milk is not desirable. However, in this study the acidity percentage of all samples from the above breeds were within the normal range.

The analyses indicated that the effects of parity, calving ages, villages, stage of lactation, and sampling time of all traits were statistically significant ($P<0.05$). However, ZAMAN *et al.* (2007) reported that the stage of lactation and parity of buffalo milk density was insignificant.

The some physicochemical compositions of Tokat Anatolian buffalo raw milk determined in this study were in agreement with other research results. It was determined that the density, acidity, urea, free fatty acids, citric acids, freezing point, and pH content of Anatolian buffalo milk were affected by various environmental factors. Additionally, the quality and chemical compositions of the milk are of great importance to the dairy sector and human health because milk composition is related to milk products.

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PAPER

EFFECT OF THYME OIL AND PACKAGING ON THE QUALITY OF SMOKED TROUT STORED AT 4°C

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ABSTRACT

In this study, the effect of thyme oil and the type of packaging on the storage of liquid-smoked rainbow trout fillets over a period of 150 d at 4°C was investigated. Fillets were subjected to microbiological (total aerobic mesophilic bacteria, psychrotrophic bacteria, lactic acid bacteria, yeast and molds), chemical (pH, thiobarbituric acid-reactive substances and total volatile base nitrogen) analyses and to sensory quality testing throughout the storage period. Bacterial growth was inhibited in samples with high concentrations of thyme essential oil due to its antimicrobial activity. Overall, the combined use of thyme EO (1%, v/w) and modified atmosphere had a synergistic effect, extending the shelf life of trout fillets.

- Keywords: rainbow trout, liquid smoking, thyme oil, shelf life, antimicrobial activity -

INTRODUCTION

Smoking is one of the oldest methods of food preservation and is widely used in fish processing (MURATORE and LICCIARDELLA, 2005; STOLYHWO and SIKORSKI, 2005). The shelf life of smoked fish products depends largely on the initial bacterial contamination level of the raw material; the water holding capacity (aw) of brined and pre-dried tissues; the activation of putrefactive microflora by heat treatment; the amount of smoke components that penetrate the product; and the temperature, air humidity and oxygen levels during storage (SIKORSKI *et al.*, 1990; IBRAHIM *et al.*, 2008). The three following methods are used to smoke fish: the traditional method of combustion at a low temperature (cold smoking ≤30°C) or a high temperature (hot smoking ≥60°C), a high-voltage electrostatic field to accelerate smoke deposition, or a liquid smoked-fish flavorant (GOULAS and KONTOMINAS, 2005). Liquid smoke flavorings are obtained from the condensation of wood smoke and are generally used as flavoring agents (SISKOS *et al.*, 2005). The ease of application, speed, product uniformity, low cost, and compatibility with the environment are among the advantages of using smoke flavorings rather than traditional smoking techniques (SIMON *et al.*, 2005; ALCICEK, 2011). The salting process is itself a preservation technique that is performed before many processing techniques (smoking, drying and marinating) are used. Salting is the first step of the fish-smoking process and is primarily used to partially dehydrate the tissue. Bacterial activity is largely prevented by high salt concentrations, and a high salt concentration prevents the growth of microflora that spoil fish, thus significantly increasing its shelf life (ISMAIL and WOOTTON, 1992). NaCl is an essential ingredient in processed meat products because it effects the water-holding capacity, prevents microbial growth, reduces water activity, facilitates the solubilization of certain proteins and enhances the flavor of processed meats by conferring a typical salty taste (ARMENTEROS *et al.*, 2009). Modified atmosphere packaging (MAP) is a form of packaging that removes air from the package and replaces the air with a single gas or a mixture of gases. The gaseous atmosphere changes continuously during storage because of the respiration of the product, biochemical changes and the slow permeation of gases through the packaging material (PARRY, 1993; IBRAHIM *et al.*, 2008). Vacuum packaging is also a type of MAP because air is removed from the package and is not replaced. In vacuum packaging, the product is normally placed in a package with low oxygen permeability, the air is evacuated, and the package is sealed. However, MAP is an increasingly popular food preservation technique. Consumer demands for fresh and convenient foods that are free of chemical preservatives has led to increased use of MAP, and this technique has the potential to reduce waste and extend the shelf life of a range of seafood (CHURCH, 1998; IBRAHIM *et al.*, 2008). The application of essential oils (EOs) has proven to be an effective preservation method that extends the shelf life of fresh foods (HARPAZ *et al.*, 2003; GIATRAKOU *et al.*, 2008; QUITRAL *et al.*, 2009; EMIR COBAN *et al.*, 2012; ERKAN, 2012). Essential oils (EOs) are aromatic, oily liquids that are obtained from plant material. Extracts from oregano, thyme, rosemary, clove, sage and mint are between the EOs used to both improve the sensory characteristics and extend the shelf life of foods. A number of EOs and some of their components have been reported to have antimicrobial activity against a wide range of spoilage and pathogenic bacteria (BURT, 2004; LAMBERT *et al.*, 2001). The aim of this study was to determine the combined effects of thyme essential oil and packaging type (vacuum and MAP) on the shelf life of refrigerated (4°C) liquid-smoked rainbow trout fillets by evaluating microbiological, chemical and sensory parameters.

MATERIALS AND METHODS

Preparation of samples

Liquid-smoked fish was prepared from rainbow trout Rainbow trout (250±25 g) were obtained from the Ataturk University Agricultural College Fisheries Department's rainbow trout breeding and research center. The fresh fish samples were carried to the laboratory and washed with tap water. A total of 132 fish samples were eviscerated, stored until rigor had resolved and then filleted, yielding a total of 264 fillets (ROBB *et al.*, 2002). The treatments included the following: V1 (control samples: vacuum packaged and liquid smoked), M1 (control samples: modified atmosphere packaged and liquid smoked), V2 (vacuum packaged with 0.1% [v/w] thyme EO added), M2 (modified atmosphere packaged with 0.1% [v/w]) thyme EO added, V3 (vacuum packaged with 0.1% [v/w]) thyme EO added) and M3 (modified atmosphere packaged with 1% [v/w]) thyme EO added. Each group included 44 fillets.

Brine salting, essential oil treatments and the smoking process

The salting and essential oil application processes were performed simultaneously. The rainbow trout fillets were immersed in brine (35% NaCl) at a ratio of 1:1 (w/w) for 1 h. Thyme essential oil was added to two lots of the filleted samples in appropriate volumes to cover both sides of each fillet using a micropipette to achieve final concentrations of 0.1 and 1% (v/wt) EO. Undiluted thyme essential oil was applied using a micropipette. In all treatments (described below),

the antimicrobials were massaged onto the product to obtain an even distribution of the oil using gloved fingers to avoid cross-contamination of the samples and the transmission of microorganisms. Liquid-smoke flavoring (GMT Food Ingredients Co., Istanbul, Turkey) (100 mL in 1 L of brine solution) was added, and fillets were dried for 30 min. After drying, the samples were smoked at 80°-90°C. The fillets were packaged by applying a vacuum or a modified atmosphere (50% CO_2 + 50% N_2).

Vacuum and modified atmosphere packaging

All of the filleted samples, including the control, were packaged in plastic bags obtained from the Südpack Verpackungen GmbH + Co (Germany). The plastic bags had the following specifications: 15x25 cm, composed of PA/PE (polyethylene/polyamide) at a thickness (3-seal bags GB 70) that allowed an O_2 permeability of 40 $cm^3/(m^2.day.atm)$ at 23°C, an N_2 permeability of $cm^3/(m^2.day.atm)$ at 23°C, CO_2 permeability of 145 $cm^3/(m^2.day.atm)$ at 23°C and a water vapor permeability of <3 g $(m^2.day.atm)$ at 23°C. After the application of essential oil and the packaging treatments were complete, the rainbow trout fillets were stored under refrigeration (4°±1°C) and were subjected to microbiological (total aerobic mesophilic bacteria, psychrotrophic bacteria, lactic acid bacteria, yeast and molds) and chemical (pH, thiobarbituric acid reactive substances-TBARS and total volatile base nitrogen-TVB-N) analyses. The microbiological and chemical analyses were performed at 0, 15, 30, 45, 60, 75, 90, 105, 120, 135, and 150 days of storage.

Microbiological analysis

A sample (25 g) was taken from each fillet, transferred aseptically to a stomacher bag containing 225 mL of 0.1% peptone water and was homogenized for 60 s using a Stomacher blender (Lab Stomacher Blender 400-BA 7021 Seward Medical, England) at room temperature. For microbial analyses, 0.1 ml samples of serial dilutions (1:10, diluent: 0.1% peptone water) were inoculated on agar plates. The total mesophilic aerobic bacteria (TMAB) and total psychrotrophic aerobic bacteria (TPAB) numbers were determined using plate count agar (PCA Merck 1.05463.0500) plates that were incubated at 30°C for 2 days or at 10°C for 7 days, respectively. The number of lactic acid bacteria (LAB) was determined using Man Rogosa Sharpe agar (MRS, de Man, Rogosa Sharpe Agar Oxoid CM0361) plates that were incubated at 30°C for 2 days. The number of yeasts and molds was determined using RBC (Rose Bengal Chloramphenicol) agar (Merck 1.00467.0500) plates that were incubated at 25°C for 5 days.

Chemical analysis

The total volatile base nitrogen (TVB-N) content was determined using the method reported by MALLE and TAO (1987). The TVB-N contents were expressed as mg/100 g of fish muscle. The thiobarbituric acid-reactive substance (TBARS) content was determined as reported by LEMON (1975) and KILIC and RICHARDS (2003). The TBARS content was expressed as μmol of malondialdehyde (MDA)/kg of fish muscle. The pH was determined according to the method of GOKALP (1999).

Sensory evaluation

Five experienced panelists who are members of the academic staff and trained in using sensory descriptors for smoked trout evaluated the quality of the rainbow trout fillets throughout the storage period. The quality of the rainbow trout fillets was assessed based on the appearance, taste and odor characteristics using a nine-point descriptive scale. A score of 7-9 indicated "very good" quality, a score of 4.0-6.9 indicated "good" quality, and a score of 1.0-3.9 denoted "spoiled." All of the samples were stored at 4°C until the sensory analyses were performed.

Statistical analysis

The experiments were replicated twice on two separate occasions using different fish samples. The analyses were performed in duplicate for each experimental replicate. All of the data obtained in this study were subjected to analysis of variance (ANOVA) followed by Duncan's multiple range test to determine the if difference between the mean values was significant at $\alpha = 0.05$ using SPSS software (SPSS, 1999).

RESULTS AND DISCUSSION

Microbiological changes

The changes in the TMAB content of the refrigerated rainbow trout fillets in vacuum packaging or MAP during the storage period are shown in Fig. 1a. On the initial day (day 0), the TMAB content (Fig. 1a) of the rainbow trout fillets was 2.0 log cfu/g. The TMAB contents of V1, M1, V2, M2, V3 and M3 rainbow trout fillets exceeded 7 log cfu/g, which is considered the upper limit for acceptable TMAB levels in fresh marine species (ICMSF, 1992) on days 90, 105, 120, 135, 135 and 150 of storage, respectively. At the end of the 150day storage period, the TMAB levels in V1, M1, V2, M2, V3 and M3 had reached 11.25, 10.23, 9.78, 9.08, 8.42 and 7.00 log cfu/g, respectively. Initially (day 0), psychrotrophic bacteria content (Fig. 1b) of the rainbow trout fillets was 2.0 log cfu/g. The psychrotrophic bac-

teria levels in V1, M1, V2, M2, V3 and M3 rainbow trout fillets exceeded 7 log cfu/g, which is considered the upper limit of acceptability of psychrotrophic bacteria in fresh marine species (ICMSF, 1992), on days 90, 105, 120, 135, 135 and 150 of storage, respectively. At the end of the storage period, the levels of psychrotrophic bacteria in V1, M1, V2, M2, V3 and M3 had reached 11.44, 10.78, 10.00, 9.54, 8.91 and 7.25 log cfu/g, respectively. Because the control group developed higher levels of total aerobic mesophilic bacteria and psychrotrophic bacteria during storage, thyme EO was observed to have an inhibitory effect on microbe growth. Microbial growth and lipid oxidation are factors that affect the shelf life and, consequently, consumers' acceptance of fresh fish and meat (ERKAN *et al.*, 2011). The control group exhibited the highest bacterial counts, and the groups treated with thyme EO exhibited the lowest bacterial counts, indicating that thyme EO has a high level of antimicrobial activity. The combination of MAP and thyme oil treatment might have a synergistic effect, hindering microbial growth and decreasing the final counts of spoilage microorganisms in rainbow trout. The rate of bacterial growth in the MAP samples was lower than that in the vacuum-packaged samples, most likely due to the presence of CO_2 gas in the packaging. Carbon dioxide can effectively inhibit the growth of total mesophilic and psychrotrophic aerobic bacteria. Several researchers have observed similar results (EMIR COBAN, 2010; ALCICEK, 2011; ERKAN, 2012; EMIR COBAN and OZPOLAT, 2013).

The initial LAB content (Fig. 1c) on day 0 was 2.0 log cfu/g. At the end of the storage period, the LAB contents of V1, M1, V2, M2, V3 and M3 were 9.02, 8.20, 6.17, 5.08, 5.28 and 4.94 log cfu/g, respectively. LAB is a facultative anaerobic bacteria that can grow under both anaerobic and aerobic conditions (PLAHAR *et al.* 1991). Similar results have been observed for smoked fish (EMIR COBAN, 2010; ALCICEK, 2011; ERKAN, 2012; EMIR COBAN and OZPOLAT, 2013).

The yeast and mold levels (Fig. 1d) were 2.0 log cfu/g on day 0. At the end of the storage period, V1, M1, V2, M2, V3 and M3 contained 7.96, 7.00, 4.94, 3.64, 3.70 and 3.28 log cfu/g yeast and mold, respectively. Similar yeast and mold contents (day 0) were reported for hot smoked fish by EMIR COBAN (2010), EMIR COBAN and OZPOLAT (2013).

Chemical changes

TVB-Nitrogen

The amount of TVB-N is an important criterion for determining the freshness of fish and fish products (KOSE and KORAL, 2005). Total volatile

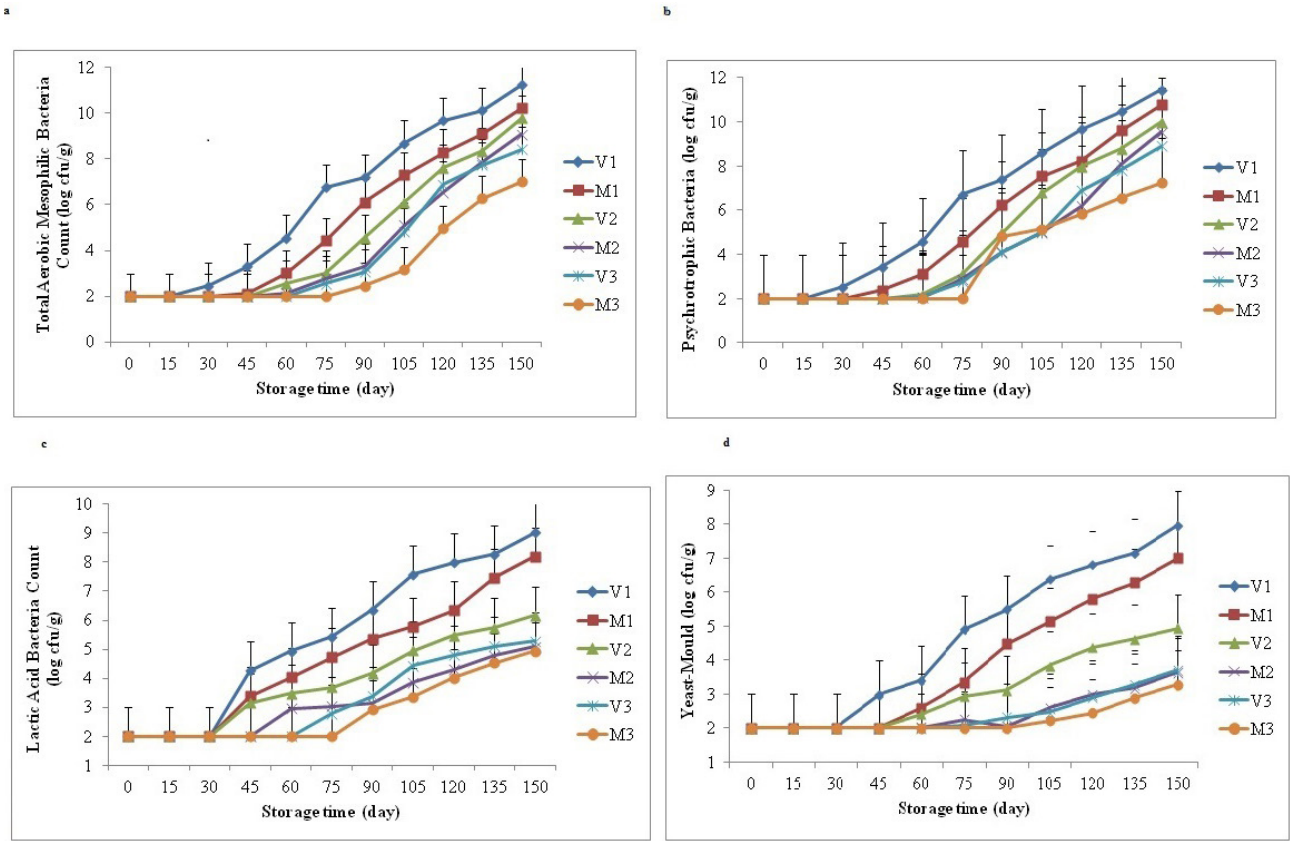


Fig. 1 - Total aerobic mesophilic bacteria counts (a), psychrotrophic bacteria (b), lactic acid bacteria counts (c), yeast and mould (d) changes of treatment with thyme essential oil (0.1% and 1 v/w) liquid smoking rainbow trout fillets during cold storage in vacuum and MAP conditions at 4°C.

basic nitrogen (TVB-N) is a product of bacterial spoilage, and the activity of endogenous enzymes and TVB-N levels are often used as an index to assess the quality and shelf life of products (EEC 1995; RUIZ-CAPILLAS and MORAL, 2005; OZOGUL *et al.*, 2006; UCAK *et al.*, 2011). In rating the quality of fresh fish, the term "very good" corresponds to a TVB-N value of up to 25 mg/100 g, "good" to values of up to 30 mg/100 g, and "marketable" to values of up to 35 mg/100 g, whereas "spoiled" denotes an unacceptable product corresponding to TVB-N values of more than 35 mg/100 g (VARLIK *et al.*, 1993). Initially (day 0), the TVB-N values (Fig. 2a) of the rainbow trout fillets were 18.12, 17.54, 15.63, 15.15, 14.26 and 12.42 for V1, M1, V2, M2, V3 and M3, respectively. The TVB-N values of the V1, M1, V2, M2, V3 and M3 rainbow trout fillets exceeded 25 mg/100 g, which is considered as the upper acceptable TVB-N limit for rainbow trout (ROBB *et al.*, 2002) on days 90, 90, 105, 105, 135 and 150 of storage, respectively. The TVB-N values of all of the groups increased during the storage period. These increases in the TVB-N value can be explained by proteolysis driven by the enzymatic and microbial activity of the samples. Values similar to our TVB-N data have been reported for rainbow trout (ALCİCEK, 2011; ERKAN, 2012).

Lipid oxidation

Lipid oxidation is one of the factors that cause product spoilage. Rancidity, a foul taste and a yellow color, might develop during the oxidation of fresh fish or fish products, particularly those with high lipid content (RUIZ-CAPILLAS and MORAL, 2001). Lipid oxidation is a major quality problem, particularly in fatty marine species. This process leads to the development of off-odors and off-tastes known as oxidative rancidity in edible oils and fat-containing foods. TBA is a secondary breakdown product of lipid oxidation and is widely used as an indicator of the degree of lipid oxidation, which can be assessed by measuring malondialdehyde (MDA) content. MDA is formed through hydroperoxides, which are the initial reaction products of polyunsaturated fatty acids and oxygen (AUBORG, 1999; FERNANDEZ *et al.*, 1998; REZAI *et al.*, 2008; UCAK *et al.*, 2011). In food suitable for consumption, the TBA values might reach the upper limit of 7 to 8 mg of MDA/kg (SINNURBER and YU, 1958; EMIR COBAN and OZPOLAT, 2013); in "perfect material," the TBA value should be less than 3 mg of MDA/kg, and in "good material," the TBA value should be no more than 5 mg of MDA/kg. The TBA values indicate the degree of rancidity of products, and values greater than 3-4 mg of MDA/kg indicate a loss of product quality (FRANGOS *et al.*, 2010). The TBARS values of the rainbow trout fillets (Fig. 2b) were 2.16, 2.06, 1.82, 1.58, 1.42 and 1.24 μmol MDA/kg initially and were 11.54, 10.08, 8.02, 7.74, 6.72 and 6.49 μmol/kg at the

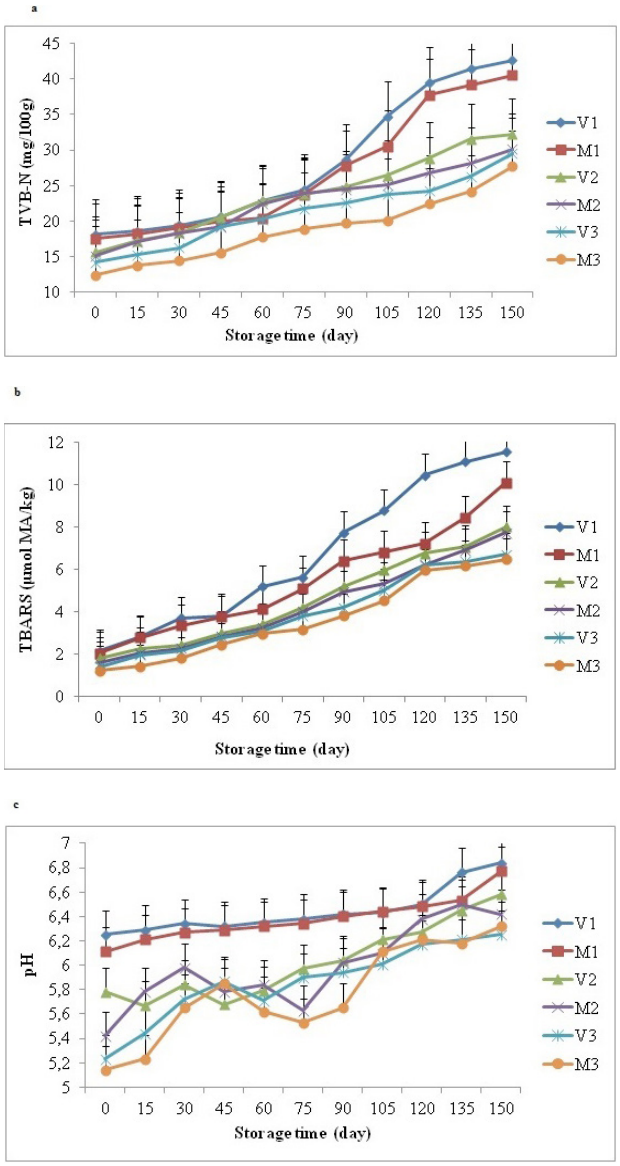


Fig. 2 - TVB-N (a), TBARS (b), pH changes of treatment with thyme essential oil (0.1% and 1 v/w) liquid smoking rainbow trout fillets during cold storage in vacuum and MAP conditions at 4°C.

end of the storage period for V1, M1, V2, M2, V3 and M3, respectively. These values increased in all groups during the storage period. Similar TBARS values have been reported for rainbow trout (EMIR COBAN, 2010; ERKAN, 2012), pike (EMIR COBAN and OZPOLAT, 2013).

pH

The pH of fresh fish flesh is approximately neutral. In the post-mortem period, the decomposition of nitrogenous compounds leads to an increase in the pH of the fish flesh, which indicates a loss of quality (CAN, 2011). The pH value of fish meat generally ranges from 5.7 to 6.6. Fresh fish has a neutral pH value, and after death, upon the formation of lactic acid, the pH value might first fall, and then rise as spoil-

age develops over prolonged storage (BILGIN *et al.*, 2007). The pH values of the rainbow trout fillets (Fig. 2c) were 6.84, 6.77, 6.58, 6.42, 6.25 and 6.32 initially and 6.84, 6.77, 6.58, 6.42, 6.25 and 6.32 at the end of the storage period for V1, M1, V2, M2, V3 and M3, respectively. Similar pH values have been reported for rainbow trout (ALCICEK, 2011; EMIR COBAN, 2010; ERKAN, 2012). The pH values of the MAP groups were lower than those of the vacuum-packed samples, which might be explained by the formation of carbonic acid from the conversion of carbon dioxide in the MAP samples.

Sensory changes

The results of the sensory evaluation (appearance, taste and odor) of the smoked rainbow trout samples are presented in Fig. 3a-c. The sensory scores for each sample indicated "good quality" after processing. However, the scores for the V1 and M1 samples had particularly decreased by the end of the storage period, which can be explained by the increase in microbial growth and TVB-N values. Similar results have been reported in other recent studies (BIRKELAND *et al.*, 2004; CARDINAL *et al.*, 2001; MARTINEZ *et al.*, 2007; EMIR COBAN 2010; UCAK *et al.*, 2011; ERKAN 2012; EMİR COBAN and OZPOLAT 2013).

Smoke and an intense thyme odor (M3) were scored by the panelists as having very good odor quality (9 points). These results showed that samples with a high level of thyme oil had acceptable overall scores due to the effect of thyme on limiting microbiological activity and decreasing the TVB-N value as well as the positive attributes of its flavor. The V3 and M3 samples were assessed by panelists as the most acceptable products.

In conclusion, adding thyme EO treatment to liquid smoking had a positive effect on the shelf life of rainbow trout fillets, maintaining the product's chemical, microbial and sensory attributes. Overall, the combined use of thyme EO (1%, v/w) and MAP exerted a synergistic effect, extending the shelf life of rainbow trout fillets.

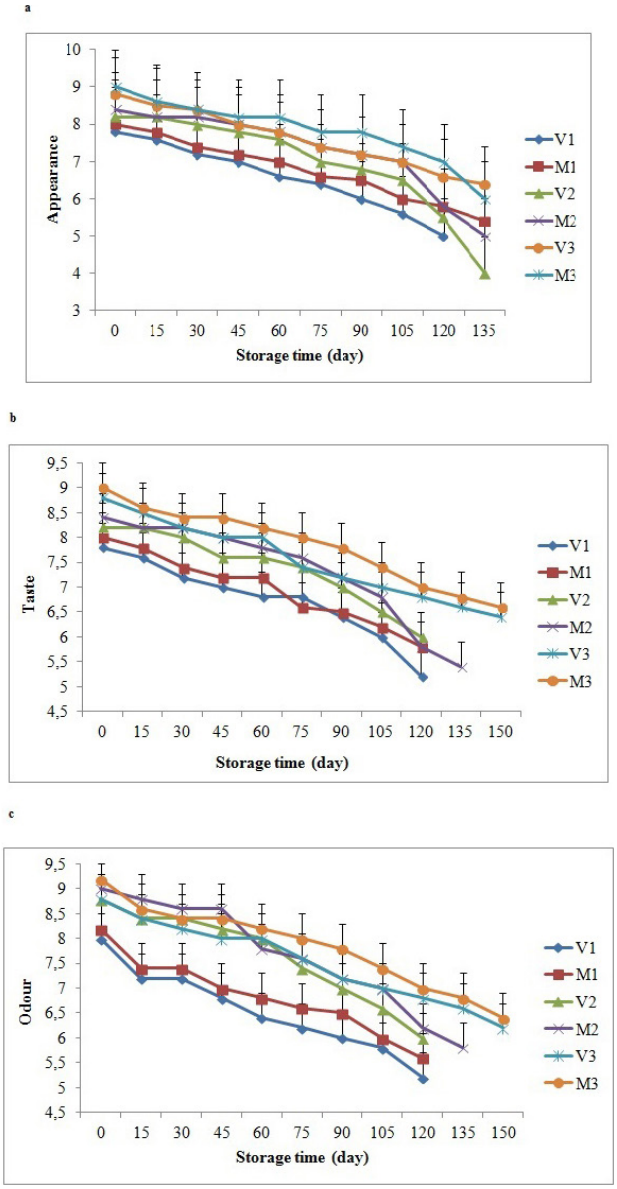


Fig. 3 - Appearance (a), taste (b), odour (c) changes of treatment with thyme essential oil (0.1% and 1 v/w) liquid smoking rainbow trout fillets during cold storage in vacuum and MAP conditions at 4°C.

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PAPER

QUALITY CHARACTERISTICS OF GROUND PORK MEAT OBTAINED USING MECHANICAL DESINEWING

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ABSTRACT

A study was conducted to characterize quality properties of ground pork meat obtained by mechanical desinewing or conventional grinding. The use of mechanical desinewing produced ground meat with higher moisture (67.4 *vs* 63.1%; P<0.01) and protein (18.7 *vs* 17.7%; P<0.01) levels and lower lipid (10.6 *vs* 14.9%; P<0.01) and collagen contents (1.89 *vs* 2.39%; P<0.01). Overall, microbial and technological properties were not modified by mincing technology. In conclusion, this study evidenced that mechanical desinewing can be effectively used to trim and mince pork meat and this enable to obtain ground meat having functional properties suitable for storage and further processing.

- Keywords: baader, desinewing; grinding; pork meat; quality -

INTRODUCTION

Over the last few decades, rapid technological progress and a greater demand for value added meat products have led to many advancements toward automation and production capacity in meat processing (BARBUT, 2014). As a consequence, there is an increasing need to also improve automation of preliminary operations involved in meat processing (i.e. boning, trimming and mincing) in order to effectively remove parts less suitable for meat product manufacturing (i.e. skin, adipose tissue and cartilagines, tendons, connective tissue layers, etc.) (MADSEN and NIELSEN, 2004). These advancements allow to reducing labour costs as well as improving processing yields and efficiency (TEMPLER, 2004; WEISS *et al.*, 2010). Some advanced technologies able to trim and mince at the same time raw deboned meats have been already available. The belt-drum system (e.g. Baader and SEPAmatic systems) was initially developed for fish, but it has been also used for pork and poultry. In this system, the tissue is passed between a rubber belt and a micro-grooved steel drum (BARBUT, 2002). Holes in the stainless steel drum range from 1 to 10 mm in diameter. Meat passes through the holes, while skin and thicker layers of connective tissue remain on the outside of the drum and are ejected through a discharge chute. Pressure on the belts can be adjusted, and sometimes, pressure rollers are used to ensure an even distribution of the tissue on the belt. The mince can range from a coarse texture to a fine paste depending on source material, machine type and setting, and processing method (FIELD, 2004; EFSA, 2013). Many studies were conducted on quality characteristics of mechanically-deboned or mechanically-separated pork meat obtained by using machines designed to recover residual meat that after manual/automated cut-up remained attached to bones (FRONING, 1981; FIELD, 1988; CROSLAND *et al.*, 1995; PÜSSA *et al.*, 2009), while few information are available on the quality characteristics of meat trimmed by machines equipped by belt-drum system (desinewing) starting from boneless raw materials. Studies conducted on other species evidenced that desinewed meat showed a dramatic reduction of connective tissue (GILLETT *et al.*, 1976; CROSS *et al.*, 1978; WELLS *et al.*, 1980; BERRY *et al.*, 1981; OSBURN and KEETON, 2004). This compositional change had obvious reflections on quality properties of finished products manufactured with desinewed meat. GILLETT *et al.* (1976) observed an increase of cooking yield, tenderness and texture in beef cooked salami when desinewed trim were incorporated. BERRY *et al.* (1981) evidenced that mechanical desinewing reduced the incidence of gelling out and in some cases improved peelability only for frankfurters made from choice minor beef cuts. Finally, OSBURN and KEETON (2004) showed that desinewing reduced cook yield of desinewed lamb trimmings.

This study was aimed to assess microbiological counts, chemico-physical and functional properties of pork meat harvested with mechanical desinewing when compared with those obtained with conventional grinder.

MATERIALS AND METHODS

Sample preparation

In order to study the effect of grinding technology on pork meat traits, three separate trials were performed. For each trial, a single batch of boned shoulders from heavy pigs (about 160 kg of live weight) was obtained at 24 h post-mortem from a local slaughterhouse and subsequently manually trimmed of part of exterior fat and connective tissue. This kind of raw materials is usually used to manufacture coarsely ground sausages marketed either fresh or after a ripening period. Within each trial, pork shoulders were divided into two aliquots and ground by conventional mincer (CM) (mod. 22-TE, Fimar, Rimini, Italy) or using mechanical desinewing (MD) (mod. 601, Baader, Brescia, Italy).

CM was equipped with a conventional system of grinding including a worm gear and a sequence of three plates and two knives to them interposed. The first plate was of "kidney" type and it was intended to reduce the size of raw materials, while the second and the third plates were equipped with holes, respectively, of 12 and 8 mm diameter. MD processing has been realized by means of a belt-drum system in which raw materials were passed between a rubber belt and a micro-grooved steel drum with holes of 8 mm diameter. Meat passed through holes, while thicker layers of connective tissue remain on the outside of the drum and were ejected through a discharge chute. During each trial, 5 samples/group of ground meat by both technologies (CM and MD) were collected and directly used to assess microbial traits. Moreover, 5 CM and MD meat samples/group were also finely minced and submitted to pH, protein functionality and proximate analyses as well as susceptibility to lipid oxidation (TBARS). Finally, 10 meat patties/group of about 80g were manufactured using each type of meat obtained by using both technologies: half patties were packed under ordinary atmosphere and stored at 2°-4°C for 96 h and used to determine colour, drip loss, and texture profile, while the second half was kept frozen at -24°C for 14 days and thawed at 2°-4°C for 48 h in order to measure thaw loss.

Analytical methods

Microbiological analyses

Ten grams of each meat sample were diluted with 90 mL of physiological solution (pH 7) and homogenised with Stomaker at normal speed for 1 min. Subsequently, 1 mL of homogenate was diluted with 9 mL of physiological solution. An aliquot of 1 mL of each serial dilution was spread onto the surface of Plate Count Agar (Oxoid) incubated for 72 hours days at 30°C to evaluate the total cell loads (UNI, 2004). *Escherichia coli* load was determined using 1 ml of diluted sample spread on Tryptone Bile Agar with X-Glucorinide (T.B.X. agar Oxoid) incubated at 44°C for 24 hours (UNI, 2010).

Proximate composition

Moisture and ashes were determined in duplicate according to the Association of Official Analytical Chemists procedure (AOAC, 1990). Proteins were determined using the standard Kjeldahl copper catalyst method (AOAC, 1990). Lipids were extracted according to a modified version (BOSELLI *et al.*, 2001) of the method described by FOLCH *et al.* (1957). Finally, collagen was determined following the modified colorimetric method of KOLAR (1990) and total collagen content was calculated by multiplying the amount of hydroxyproline by 7.5.

Determinations on meat patties

The CIE (1976) system colour profile of lightness (L*), redness (a*), and yellowness (b*) was measured by a reflectance colorimeter (Minolta Chroma Meter CR-400, Minolta Italia S.p.A., Milano, Italy) using illuminant source C, a standard observer at 10° and aperture size of 25 mm. The colorimeter was calibrated throughout the study using a standard white ceramic tile (reference number 1353123; Y = 92.7, x = 0.3133, and y = 0.3193). Colour was measured on triplicate on the surface of each meat patty at 48 and 96 hours of refrigerated storage at 2-4°C.

Drip loss was measured on each meat patty kept through refrigerated storage (0, 48 and 96h) and calculated as percentage of weight loss (HONIKEL, 1998).

Textural parameters were determined on core samples after cooking (3 cm diameter, 0.8 cm height), axially compressed (50 kg load cell; crosshead test speed 1 mm/s) to 50% of their initial height in a double compression cycle: hardness (kg, maximum force required to compress the sample), cohesiveness (A2/A1, extent to which the sample could be deformed prior to rupture, where A1 represents the total energy required for the first compression and A2 the total energy required for the second compression), springiness (D2/D1, the ability of sample to recover its original form after the deforming force is removed where D1 represents the initial compression distance and D2 the distance detected for the second compression), gumminess (hardness × cohesiveness, the force needed to disintegrate a semisolid sample to a steady state of swallowing), chewiness (springiness × gumminess, the work needed to chew a solid sample to a steady state of swallowing) (LYON and LYON, 1990).

For determination of thaw loss, meat patties were frozen at -24°C and after 14 d placed on a 2 to 4°C cooler for 48 h to allow for a slow thaw period. Samples were then blotted dry and weighed to determine thaw losses (HONIKEL, 1998).

pH

The pH was determined using a modification of the iodoacetate method initially described by JEACOCKE (1977). Approximately 2.5 g of minced meat was homogenized in 25 mL of a 5 mM iodoacetate solution with 150 mM potassium chloride for 30 s, and the pH of the homogenate was determined using a pH meter calibrated at pH 4.0 and 7.0 equipped with 924001 electrode (Bibby Scientific Ltd, T/As Jenway, Essex, UK).

Protein functionality

The centrifugation method was applied for evaluating the water binding capacity according the procedure of HONIKEL and HAMM (1994). Exactly 10.0 g of ground meat was added with 20 mL of a 5% NaCl solution in a 50 mL centrifuge tube which was centrifuged at 3,500 × g for 15 min at 6 to 8°C just after a storage period of 30 minutes at 2-4°C. The supernatant was discarded, the tube was thoroughly drained, and the weight of the pellet was determined and swelling was expressed as gained weight (%) based on initial weight. The tubes were subsequently heated at 80°C for 20 min on a water bath. After incubation, the released water was discarded, and the tubes were allowed to equilibrate to room temperature and reweighed for cooking loss determination

TBARS analysis

The susceptibility of muscle tissue homogenates to iron-induced lipid oxidation was determined according to the method of KORNBRUST and MAVIS (1980). Tubes containing 100 µl of homogenates were incubated at 37°C and removed at fixed time intervals (0, 30, 60, 90, and 150 min) for measurement of 2-thiobarbituric acid-reactive substances (TBARS). Protein content of the meat was determined according to the Lowry procedure (LOWRY *et al.*, 1951) and TBARS expressed as nmoles malonaldehyde (MDA)/mg protein.

Statistical analysis

Data were analyzed using the ANOVA option of the general linear models (GLM) procedure of SAS software (SAS Institute, 1988). The model tested the main effects for meat harvesting (CM *vs* MD) and trial (1, 2 and 3), as well as the interaction term, using residual error. Drip loss, colour coordinates (L*a*b*) and TBARS data were analyzed using a mixed model ANOVA with repeated measures with meat harvesting and trial as the main effects

RESULTS AND DISCUSSION

Total microbial viable count were in the normal range of ground pork meat and were significantly lower in samples yielded with MD, even if absolute difference was of relatively little practical importance (Table 1) and substantially lower than limits ruled by Regulation 2073/2005. FIELD *et al.* (1974) found no differences in bacterial counts/gram of meat from hand- or machine-boned lamb carcasses used to make bologna, while OSBURN and KEETON (2004) found that aerobic plate count was slightly lower in desinewed meat in respect to conventionally ground lamb meat. The initial microbial load present on the desinewing machine and grinder used in meat comminuting, as well as possible variations in the time required to debone the respective pork cuts may also be factors contributing to the differences in total microbial count. Furthermore, *E. coli* is considered as indicator of the hygienic conditions under which minced meat is handled and in our research all samples from both groups resulted below to the detection limit (10 CFU/g) (Table 1).

As regard with chemical composition (Table 2), the use of MD produced meat with higher moisture (67.4 *vs* 63.1%; P<0.01) and protein (18.7 *vs* 17.7%; P<0.01) levels and lower lipid (10.6 *vs* 14.9%; P<0.01) and collagen contents (1.89 *vs* 2.39%; P<0.01). All these differences are likely attributable to the removal of the of ticker connective tissue layers (e.g. epymisium) and part of adherent subcutaneous fat depots which determined a reduction of collagen and lipid content with a consequent increase of moisture and total proteins in minced meat harvested using MD in respect to CM. These results fully agree with GILLETT *et al.* (1976) who found that mechanical desinewing removed approximately half the connective tissue by increasing protein and moisture content and by reducing fat level of ground beef meat. All previous studies showed that mechanical desinewing was effective in removing connective tissue (GILLET *et al.*, 1976; WELLS *et al.*, 1980; BERRY *et al.*, 1981; OSBURN and KEETON, 2004). In this regard, it is interesting to note that the EC Regulation 2076/2005 requires the compulsory indication on the label of the minced meat of the collagen/protein ratio which cannot exceed the different limits of 18 for meats by imposing constraints to the use of raw materials containing high amounts

Table 1 - Microbial traits of ground meat obtained using conventional mincer (CM) and mechanical desinewing (MD) (n=30/group).

Parameter	Technology		esm	Probability
	CM	MD		
Total microbial count (Log UFC/g)	3.5	3.3	0.06	**
E. coli (Log UFC/g)	not detected[a]	not detected[a]	-	-

** = P<0.01
[a]= below to detection limit (10 CFU/g)

Table 2 - Proximate composition of ground mea obtained using conventional mincer (CM) and mechanical desinewing (MD) (n=30/group).

Parameter	Technology		esm	Probability
	CM	MD		
Moisture (%)	63.1	67.4	0.59	**
Protein (%)	17.7	18.7	0.97	**
Lipid (%)	14.9	10.6	0.61	**
Collagen (%)	2.39	1.89	0.33	**

** = P<0.01

Table 3 - pH value and protein functionality (swelling and cooking loss) of ground meat obtained using conventional mincer (CM) and mechanical desinewing (MD) (n=30/group).

Parameter	Technology		esm	Probability
	CM	MD		
pH	5.93	5.95	0.01	ns
Swelling (%)	42.0	44.2	0.96	ns
Cooking loss (%)	37.6	38.9	0.36	ns

ns = not significant

of connective tissue. Indeed, MD technology allowed to extensively reduce collagen/protein ratio from 13.5 to 10.8.

The pH values were in the normal range of ground pork meat (FERNÁNDEZ-LÓPEZ *et al.*, 2004) and, as expected, there were no significant differences between groups because the origin of raw materials was the same (Table 3). This consistency is important because discrepancies in colour and water holding capacity are strictly connected with differences in pH (BROWN *et al.*, 2000; BARBUT *et al.*, 2008). No differences were detected on swelling and subsequent cooking losses which were measured as indicators for protein functionality (Table 3). Also loss of liquid during refrigerated storage (drip loss) assessed on meat patties did not differ after 48 and 96 h (Fig. 1). On the other hand, losses of liquids after thawing (thaw loss) was lower in MD patties (Fig. 1). This latter result may be attributed to the higher concentration of proteins found in MD minced meat which increases the ability of the meat to retain liquids, as it is closely related to the amount of proteins (HUFF-LONERGAN and LONERGAN, 2005). It is well-known that frozen storage can decrease solubility and increase oxidation of the proteins (LEYGONIE *et al.*, 2012) and this may explain lacking of difference on protein functionality and drip loss of fresh meat in respect to liquid losses after freezing/thawing phases which can have stressed differences between samples. It is noteworthy to note that, with the current state of the market, freezing has come to play a tremendous role to both store and trade of raw materials, because frozen storage constitutes the most effective means of maintaining high-quality and safe meat products during storage for producers, distributors, catering and final consumers (RESURRECCION, 2004).

It is well-known that minced meat easily undergoes discoloration because of oxidation of ferrous myoglobin to ferric iron met-myoglobin and these influences the choice of the consumer at the time of purchase (GRAY *et al.*, 1996; MANCINI and HUNT, 2005). In Fig. 2, onset of redness (a*) assessed on meat patties packaged under ordinary atmosphere and stored at 2°-4°C for 96 hours has been shown. In both groups, as expected redness gradually decreased over time because of the changes from red to brown due to oxidizing oxymyoglobin to metmyoglobin, but no significant differences between groups were detected. This result is in agreement with the finding reported by CALHOUN *et al.* (1999) who did not reveal substantial differences in the colour changes of hamburgers prepared with meat boned and manually trimmed and then minced using conventional procedures with those obtained from advanced recovery systems.

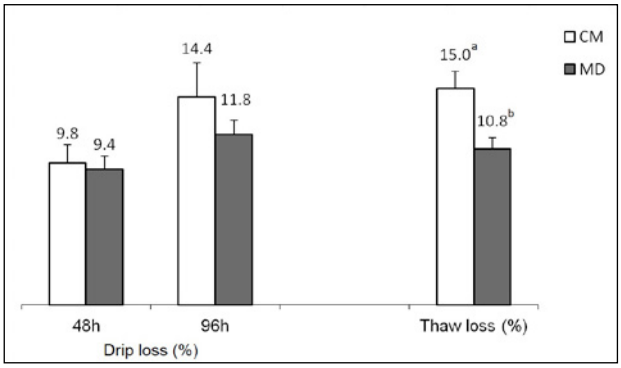


Fig. 1 - Drip and thaw losses (mean±SEM) in raw patties manufactured with meat obtained using conventional mincer (CM) and mechanical desinewing (MD) (n=30 group; a-b=P<0.05).

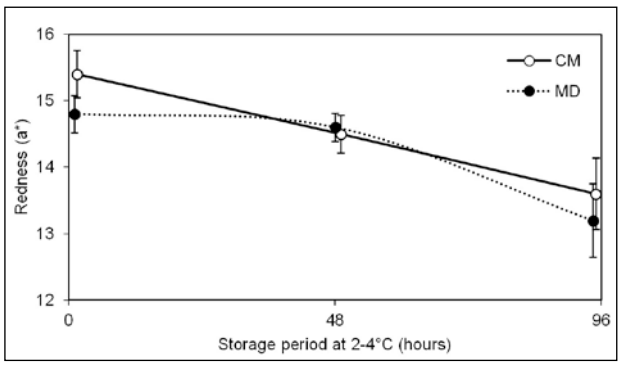


Fig. 2 - Evolution of redness values (a*, mean±SEM) in raw patties manufactured with meat obtained using conventional mincer (CM) and mechanical desinewing (MD) and packaged under ordinary atmosphere for 96 h at 2-4°C (n=30/group).

Table 4 - Texture profile of cooked patties manufactured with meat obtained using conventional mincer (CM) and mechanical desinewing (MD) (n=30/group).

Parameter	Technology		esm	Probability
	CM	MD		
Hardness (kg)	8.41	10.09	1.10	**
Cohesiveness	2.46	2.12	0.30	**
Gumminess (kg)	30.6	21.4	1.78	ns
Springiness	1.30	1.26	0.04	**
Chewiness (kg)	26.8	26.9	2.22	ns

** = P<0.01; ns = not significant

Texture of meat patties after cooking showed several significant differences (Table 4). Samples obtained with the mechanical desinewing presented higher values of hardness and lower levels of cohesiveness and elasticity, while chewiness and gumminess showed no significant differences. These results prove that the grinding technology exerted a substantial influence on the texture of the meat after cooking, however these differences may be mainly attributed to changes in chemical composition. Indeed, it is well-know that protein concentration is the major factor in determining desired gel strength in comminuted products (ASGAR *et al.*, 2010), as a consequence a reduced collagen level in MD raw materials can have determined a decrease of instrumental hardness assessed by TPA in MD patties. Otherwise, differences observed in springiness and cohesiveness may be attributed to the differences in lipid level found between MD and CM raw materials used to manufacture meat patties. Springiness values are related to the elastic properties of the patty, where a decrease in the springiness value indicates that the elasticity of the patty decreased. Lower springiness found in patties manufactured with MD may be attributed to the lower fat content. This result was not in agreement with that JUNG and JOO (2013) who reported that fat reduction result in an increase in springiness in pork patties. Previous authors found that springiness decreased when the fat level was increased (CREHAN *et al.*, 2000). Cohesiveness, a measure of how good the sample retains its structure after compression, was found to be lower in MD patties. As for springiness, this effect may be attributed to the lower content of fat observed in raw materials which has been demonstrated to reduce cohesiveness of pork patties (CHOI, 2012) and meat balls (ULU, 2006). In a previous study, CALHOUN *et al.* (1999) found that some rheological properties of cooked burgers manufactured with meat from advanced recovery systems were worse (less consistency and cohesion). However, CALHOUN *et al.* (1999) used an advanced system to recover meat from bone-in raw materials, so it can be argued that the working pressure was considerably higher than those used in the present study. This may have resulted in a greater degree of destruction of the muscular structures that may have a negative effect on the texture of the final product.

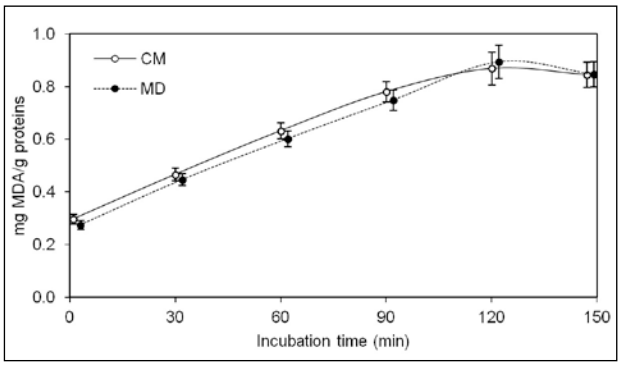


Fig. 3 - Lipid susceptibility to oxidation (TBARS, mean±SEM) of ground meat obtained using conventional mincer (CM) and mechanical desinewing (MD) (n=30/group).

Finally, grinding technology did not influence the susceptibility to lipid oxidation (Fig. 3). It is well-known that any process causing disruption of the membranes such as size reducing processes (grinding, flaking, mincing, etc), deboning, and cooking results in exposure of the phospholipids to oxygen, and, therefore, accelerates development of oxidative rancidity (St. ANGELO, 1996). Despite the different grinding technologies used for yielding minced meat adopted in the present study, susceptibility to lipid oxidation did not differ between groups.

CONCLUSIONS

In conclusion, this study evidenced that mechanical desinewing can be effectively used to trim and mince different pork meat by automatically removing parts rich of connective tissue (tendons, cartilages and thick collagen layers) and it allow to obtain minced meat having microbiological traits and func-

tional properties suitable for storage and further processing.

The adoption of mechanical desinewing may increase the degree of automation in meat processing with possible positive effects not only on the reduction of labor cost but also on improving production capacity and uniformity.

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PAPER

EFFECT OF PIG SLAUGHTER WEIGHT ON CHEMICAL AND SENSORY CHARACTERISTICS OF TERUEL DRY-CURED HAM

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ABSTRACT

A preliminary study was carried out with 36 barrows to investigate the effect of slaughter weight (SW; 120, 130 and 140 kg) on chemical, instrumental and sensory characteristics of Teruel dry-cured ham. The intramuscular fat content tended to increase and salt, potassium nitrate and sodium nitrite contents decreased as SW increased. The panelists detected wider subcutaneous fat and lower cured colour, saltiness, hardness and fibrousness in hams from heavier pigs but no difference was observed on overall quality assessment. In conclusion, pig SW affected some chemical and sensory traits of dry-cured ham, which contributes to increase the heterogeneity.

- Keywords: slaughter weight; chemical characteristics; sensory quality; dry-cured ham; heavy pigs -

INTRODUCTION

Spain is the world leader in dry-cured ham and shoulder production reaching annually 40 million pieces (MAGRAMA, 2013). The Protected Designation of Origin (PDO) "Teruel ham" was the first Spanish PDO to control and guarantee the ham production. It establishes several requirements to try to ensure uniformity and quality in the end product (BOA, 2009). Some of them are related to the pig management (crossbreed Duroc x (Landrace x Large White) and slaughter weight (SW) between 120 and 140 kg), others are checked at the abattoir (carcass weight >84 kg, fat thickness over the *Gluteus medius* muscle >16 mm and fresh ham weight >11.5 kg) and others are concerning to the dry-curing process (length >18 months, weight loss of ham = 35-40% and cured ham weight >7 kg).

In spite of fulfil it, a great heterogeneity among pieces of Teruel ham is observed even when they come from the same cellar. One of the main problems manifested by the Teruel ham industry is the wide range of piece weights due to the different pig SW which carry out differences in the quality. To our knowledge, the information about the effect of the pig management on quality of this kind of ham is quite limited because most of experiments have been focused on fresh pork extrapolating the results to the cured product (LATORRE et al., 2009a and 2009b).

Therefore, the aim of this study was to investigate the effect of pig SW (120, 130 and 140 kg) on some instrumental, chemical and sensory characteristics in Teruel dry-cured ham.

MATERIAL AND METHODS

Slaughter of experimental pigs

A total of 36 Duroc x (Landrace x Large White) barrows were used in a preliminary study. There were three experimental treatments in base on preplanned SW of pigs: 120, 130 and 140 kg (118.9 ± 3.21 kg, 128.7 ± 3.53 kg and 139.2 ± 3.62 kg as average; and 189, 203 and 217 ± 3 days of age, respectively), which fulfilled the *Consortium* rules of the PDO Teruel ham. Animals belonged to the same farm and the previous feeding and management was exactly the same for all of them. At the abbatoir (Jamones y Embutidos Altomijares, S.L., Formiche Alto, Teruel, Spain), pigs were slaughtered and carcasses were processed according to standard commercial procedures (the whole hams were taken from the carcass, with no cut between hind shank and leg, because were intended for dry-curing process). After fitting market demands (round shape), hams were trimmed, removing part of external fat, and individually weighed.

Dry-curing process and sampling of hams

The left ham from each carcass was subjected to the drying process according to the *Consortium* rules of Teruel ham (BOA, 2009). Briefly, the femoral artery of the pieces was manually pressed in order to purge the blood residues and reduce the risk of spoilage later on. At 3 days *postmortem*, the salting period started. For it, hams were coated with a mixture (0.3 g $NaNO_2$, 0.4 g KNO_3 and 10 g NaCl per kg of raw ham) and kept at 2°-4°C and 80-90% relative humidity for a variable time depending on the green ham weight (1 day/kg of ham). The next step was washing hams with cold water to remove the excess salt before starting the post-salting period. For it, pieces were stored at 3°-6°C and 80-90% relative humidity for 60, 70 and 80 days for pigs slaughtered at 120, 130 and 140 kg, respectively. During the last stage (ripening period), hams were dried increasing the temperature (from 10° to 30°C) and decreasing the relative humidity (from 85 to 70%). The process was finished when total weight loss of pieces reached 35-40% of initial weight. The individual weight of hams was recorded throughout the process to calculate the weight loss by phase.

When dry-cured period ended, a total of 12 processed hams were randomly selected (4 hams per each SW). The limited number of replicates was due to the preliminary nature of the study. From each ham, a sample of 600±55 g and 20 mm thick was taken transversally, as is described in Fig. 1, and was intended for the chemical analyses. Other sample of 1,100±60 g from the central part ("maza") was taken (Fig. 2) and was intended for the analysis of instrumental colour and sensory evaluation. All samples were moved to the laboratory and were vacuum packaged and stored at 4°±2°C in darkness during one week until the analyses.



Fig. 1 - Cross-section of a dry-cured ham intended for chemical analyses.



Fig. 2 - Sample of a dry-cured ham intended for colour measures and sensory analysis.

Chemical analyses and instrumental measure of colour

The chemical analyses were carried out in the *Semimembranosus* (SM) muscle of each sample because it is the reference muscle in the PDO Teruel ham (BOA, 2009). Moisture content was determined by drying at 103°± 2°C to a constant weight (BOE, 1979) and Folch method (FOLCH *et al.*, 1957) was employed for determining intramuscular fat (IMF) content. To estimate the salt content, chlorides were extracted with water–ethanol (60:40 v/v) and quantified by the Carpentier-Volhard method (AOAC, 1984). To determine the nitrites content, sodium nitrite was measured by absorption spectrophotometry at 520 nm using an equipment HITACHI model U-1100 (USA). Also, the nitrates content was obtained measuring the potassium nitrate using the same spectrophotometer at 410 nm.

From "maza" samples, colour was measured on *Biceps femoris* (BF) and SM muscles and also on subcutaneous fat by a chromameter (CM 2002, Minolta Camera, Osaka, Japan), previously calibrated against a white tile according to manufacturer recommendations, using objective measurements (CIE, 1976). The average of three random readings was used to measure lightness (L*, a greater value is indicative of a lighter colour), redness (a*, a greater value is indicative of redder colour), and yellowness (b*, a greater value is indicative of a more yellow colour). Additionally, chroma (C*) and hue angle (H*) were calculated as $C^* = \sqrt{(a^{*2} + b^{*2})}$ and as $H^\circ = \tan^{-1}(b^*/a^*) \bullet 57.29$, respectively. Chroma is related to the quantity of pigments and high values represent a more vivid colour denoting lack of greyness, and H° is the attribute of a colour perception denoted by blue, green, yellow, red, purple, etc. related with the state of pigments (WYSZCECKI and STILES, 1982).

Evaluation of sensory characteristics

Ham samples were assessed by a trained panel of 10 members (ISO 8586-1, 1993). To acquaint panelists with product attributes and intensities, six 1 h training sessions took place over a 4-week period prior to sample testing. The sensory analysis was performed in individual cabins under controlled environmental conditions and a red light to obscure meat colour (ISO 8589, 1988). The panel sessions were held at mid-morning, about 3 h after breakfast. Slices (1.5 mm) of the "maza" were obtained with a slicing machine about 1 h before tasting, in order to allow slices to reach room temperature (22°C). They were served on plates to panelists, which were told to taste narrow slice sections including both BF and SM muscles.

A profile of 18 sensory attributes of dry-cured ham was assessed. Attributes were grouped in appearance, odour, texture, flavour and acceptability. For evaluating overall quality, panel members were asked to give a semihedonic quality score, based on their expertise in integrating ham sensory attributes into a conclusive quality value. Attributes were rated on a 9-point structured scale (1 = very low to 10 = very high). About 50 ml of water at room temperature and 20 g of unsalted bread were provided between successive hams. A total of four sessions were carried out at 22°C in a sensory panel room equipped with white fluorescent lighting (Philips TLD 86, 5600°K, 800 lux). The sample order was randomized within assessors and sessions.

Statistical analyses

All data were analysed as a completely randomized design using the General Linear Model procedure of SAS (1990). Briefly, the Normal distribution for all variables was checked. The statistical model included the SW (120, 130, and 140 kg) as main effect. Additionally, when significant differences were detected, linear effects were evaluated. For the sensory data, a previous GLM procedure of SPSS (2005) was performed including the session and SW for each panelist as fixed effect. Afterwards, another GLM was performed with the mean per attribute and per SW obtained from the previously corrected data file. Duncan's test was used to compare means where the variance analysis indicated a significant effect. The experimental unit was the animal (n=12 for ham weight losses and n=4 for chemical, instrumental and sensory traits). A value of $P<0.05$ was used to assess the significance, whereas a P-value between 0.05 and 0.10 was classified as a trend.

RESULTS AND DISCUSION

Weight loss of hams during dry-curing process

As it was expected, the ham weight, either fresh or dry-cured, increased linearly as pig SW increased (P<0.001) (Table 1). The rate of increase in fresh weight was 1.28 kg and in dry weight 1.15 kg per each 10 kg SW of pigs. The difference between both (approx. 10%) would indicate that heavier hams had lower weight loss through the drying process than lighter hams. In fact, when this variable (total loss) was calculated, a linear reduction by 2.6 percentage units for every 10-kg increase above 120 kg was observed (P<0.001). The processing length was shorter in lighter than in heavier hams (72, 76, and 80 weeks for 120, 130 and 140 kg SW, respectively) to fulfil the requirement about total loss (35-40%) established by the *Consortium*. In summary, pig SW affects fresh ham weight which, in turn, influences process technology such as the salting, post-salting and ripening length and carries out differences in final weight losses of product.

The main differences among treatments in the ham weight loss happened at the beginning of the process. In fact, during salting and post-salting periods, linear and significant reductions in ham weight loss were observed (1.1 and 1.5 percentage units per each 10 kg of pig SW, respectively; P<0.001). The weight loss during the first stages is due to water that dissolves the salt and intensively drips out of ham. In the later periods of processing, the weight loss is because of evaporation from the surface and an equilibration inside the ham (TOLDRÁ, 2002). In the current trial, a decrease in ham weight loss during the ripening period was found as pig SW increased, although it was only numerical (18.9, 18.5 and 18.3% for 120, 130, and 140 kg SW, respectively). CANDECK-POTOKAR and SKRLEP (2012) reported that one of the factors with highest influence on processing losses, especially in that phase, was the fat thickness which serves as a barrier for water evaporation. Although cover fat depth was not measured instrumentally, the visual evaluation of panelists confirmed that it was wider in heavy than in light pigs. The ripening is the longest period and therefore a higher ham weight loss was found during that stage (18.5% as average) whereas it was lower for salting and post-salting phases (7 and 12% as average, respectively). A high variability of data was observed during the ripening period and it might explain, at least in part, the lack of significant influence of the ham weight in that phase. The values of ham weight loss through the processing were similar to those obtained by PEINADO et al. (2005) in hams from Pietrain-sired pigs slaughtered with 122 kg.

Chemical analyses and instrumental colour

The chemical analyses of dry-cured ham showed values (/100 g wet matter) of moisture from 47.0 to 50.0 g and of IMF from 12.5 to 13.5 g (Table 2). Those results are close to those found by GOU *et al.* (2008) in hams with regular pH values. Although it was not significant, the moisture content seems to be lower in heavier hams, probably due to the longer processing length and/or the higher fat content of the piece. Also, TIBAU et al. (2002) detected less moisture in the carcasses from older and heavier pigs, suggesting that the activity of hydrolytic enzymes was reduced in cured hams. The IMF content, detected in the trial, tended to increase linearly (by 0.438 g/100 g per each 10 kg; P<0.10) as pig SW increased. The positive correlation between IMF of pork and SW of pigs has been widely demonstrated in the literature (WEATHERUP *et al.*, 1998; LATORRE *et al.*, 2009b). The different pig age determines a high variability in the composition characteristics of

Table 1. The effect of pig slaughter weight on weight loss of hams during the dry-curing process.

	Slaughter weight, kg			SEM [a]	R^2	Slope	P [b]
	120	130	140				
Ham weight, kg							
Fresh	11.9z	13.5y	14.5x	0.29	0.45	+0.128	***
Dry-cured	7.1z	8.4y	9.4x	0.22	0.54	+0.115	***
Weight loss during dry-curing process, %							
Salting	8.2x	6.7y	6.2y	0.32	0.33	-0.11	***
Post-salting	13.3x	12.4xy	10.3y	0.49	0.37	-0.15	***
Ripening	18.9	18.5	18.3	0.61			NS
Total	40.4x	37.6y	34.8z	0.94	0.34	-0.26	***
Dry-curing process length, weeks	72	76	80				

[a] SEM: standard error of the mean (n=12).
[b] P: level of statistical significance. NS: P>0.10; ***P<0.001. x,y,z Within a row, means without a common superscript letter differ (P<0.05).

Table 2. The effect of pig slaughter weight on some traits related to chemical composition of dry-cured hams [a].

	Slaughter weight, kg			SEM [b]	R^2	Slope	P [c]
	120	130	140				
Moisture, g/100 g	49.97	47.32	47.12	1.202			NS
Intramuscular fat, g/100 g wet matter	12.54	13.20	13.42	0.511	0.21	+0.044	†
Sodium chloride, g/100 g wet matter	5.74	5.30	4.71	0.284	0.31	-0.0511	†
Potassium nitrate, mg/kg wet matter	2.23x	1.23y	1.01z	10.172	0.29	-0.061	**
Sodium nitrite, mg/kg wet matter	153.6x	98.5y	67.8z	0.17	0.28	-4.262	**

[a] Measured on *Semimembranosus* muscle.
[b] SEM: standard error of the mean (n=4).
[c] P: level of statistical significance. NS: P>0.10; †P<0.10; **P<0.01. x,y,z Within a row, means without a common superscript letter differ (P<0.05).

muscle because older pigs had more time for fat retention. In turn, different chemical composition carries out differences in other variables as is described as follows.

The sodium chloride content tended to be reduced in almost 1 percentage point (P<0.10) as SW of pigs increased from 120 to 140 kg which indicates a less amount of salt absorbed by heavier hams. The values detected for it ranged from 4.7 to 5.7 g/100 g wet matter which are normal for this kind of product and similar to those found by GARCÍA-REY *et al.* (2004) and GARCÍA-GIL *et al.* (2012) although these authors concluded that it also depends on other factors such as pH, skin trimming or pressing. It is necessary to ensure a minimum salt content in hams to maintain microbiological stability and to avoid excessive softness (VIRGILI *et al.*, 1995; RUÍZ-RAMÍREZ *et al.*, 2006). On the other hand, an excessive salt content causes undesirable saltiness and contributes to increased risk of high blood pressure (MORGAN *et al.*, 2001). In the present study, the trend to a lower salt content as pig SW increased is positive under a healthy point of view because nowadays low salt levels are recommended in human diet (ARMENTEROS *et al.*, 2012).

The potassium nitrate and sodium nitrite contents decreased (P<0.01) as pig SW increased. The addition of nitrificant salts to dry-cured ham reinforces the preservative effect of salt by inhibiting the growth of *Clostridium botulinum*. Nitrite, which is the active form, also contributes to the development of dry-cured ham flavour and to the formation of the characteristic reddish colour in the final product. However, a compromise between microbiological and toxicological safety must be achieved (TOLDRÁ *et al.*, 2009). Low levels of nitrite in the end product are recommended to minimize the possibility of nitrosamines generation. The reduction in NaCl, $NaNO_2$ and KNO_3 contents might be related to the size or weight of hams. It can be possible that the diffusion of these chemical compounds in the aqueous phase of muscle in heavier pieces will be less intensive and thus heavier hams exhibit lower contents than those lighter.

No effect of treatment was observed on colour of dry-cured ham measured at BF and SM muscles or at subcutaneous fat (P>0.10) (data not shown). The values of L*, a* and b* were similar to those reported by CILLA *et al.* (2006) obtained in hams with the same curing length from pigs of the same crossbred and SW. However, the influence of pig SW has been more deeply evaluated on colour of fresh meat than on that of dry-cured products being the results always subject of debate. As SW increased, some authors (GARCÍA-MACÍAS *et al.*, 1996; LATORRE *et al.*, 2004) found a redder colour and with high myoglobin content in *Longissimus dorsi* muscle whereas other authors (UNRUH *et al.*, 1996; WEATHERUP *et al.*, 1998) observed that colour was independent of SW. In the case of dry-cured products, such as ham, other factors can have more influence on colour than pig SW, i.e. the use of salt nitrite during the processing. In spite of the effect found on sodium nitrite content in the present trial, a lack of influence was observed on colour traits measured by a Minolta chromameter at BF and SM muscles, and also at subcutaneous fat. However, the panelists detected lower cured colour in hams from heavier pigs which might be related, in part, to the low nitrite content detected.

Sensory characteristics

The cured colour of ham decreased in BF (P<0.01) and in SM (P<0.05) muscles as pig SW increased (Table 3). Besides the nitrite content, other factors can affect the colour of cured ham, such as the dry-curing length. In fact, CILLA *et al.* (2005) found, in hams of 8.5-9.5 kg, that the cured colour of BF muscle increased as the processing length increased from 12 to 26 months. Probably, in the present trial, the difference in the length of processing (from 72 to 80 weeks) was not enough to cause a detectable effect.

In addition, panelists observed higher subcutaneous fat whereas SW increased (P<0.001), in agreement with results of several authors who

Table 3. The effect of pig slaughter weight on some sensory characteristics of dry-cured ham [a].

	Slaughter weight, kg			SEM [b]	P [c]
	120	130	140		
Appearance attributes					
Cured colour at *Biceps femoris* muscle	5.89x	5.72x	5.06y	0.173	**
Cured colour at *Semimembranosus* muscle	7.19x	6.86xy	6.61y	0.158	*
Colour homogeneity	5.64	6.00	5.50	0.223	NS
Intramuscular fat at *Biceps femoris* muscle	7.06	7.23	7.33	0.220	NS
Intramuscular fat at *Semimembranosus* muscle	5.92	6.06	6.31	0.221	NS
Subcutaneous fat	5.31x	6.41y	6.44y	0.185	***
Visual defects	1.64	2.00	1.72	0.172	NS
Odour attributes					
Aroma	6.11	5.94	6.25	0.181	NS
Odour defects	1.64	1.56	1.34	0.189	NS
Texture attributes					
Hardness	5.50x	5.00xy	4.58y	0.256	*
Crumbliness	5.56	5.58	5.75	0.268	NS
Pastiness	2.78	3.11	3.58	0.276	NS
Fibrousness	3.51x	3.00xy	2.33y	0.291	*
Flavour attributes					
Flavour	6.08	5.89	5.75	0.182	NS
Saltiness	5.64x	5.44xy	4.94y	0.179	*
Rancid flavour	1.31	0.92	0.94	0.152	NS
Flavour defects	1.33	1.68	1.33	0.205	NS
Acceptability					
Overall quality assessment	6.28	6.22	6.17	0.191	NS

[a] Measured by a numerical scale (1-10).
[b] SEM: standard error of the mean (n=4).
[c] P: level of statistical significance. NS: P>0.10; *P<0.05; **P<0.01; ***P<0.001. x,y Within a row, means without a common superscript letter differ (P<0.05).

measured it instrumentally (WEATHERUP *et al.*, 1998; LATORRE *et al.*, 2009b). The increased adiposity could limit their marketability. In spite of those findings in covering fat and the trend to a higher IMF content detected analytically, the sensory study showed only a numerical effect (P>0.10) on IMF observed by panelists. LATORRE *et al.* (2004) reported that fat thickness at level of *Gluteus medius* muscle, which is covering the ham, increased by 2.3 mm for each 10 kg increase in SW above 116 kg but these authors neither find any influence on IMF. It would confirm the study of HUFF-LONERGAN *et al.* (2002) who reported a positive linear but moderate correlation (r=0.45) between backfat depth of carcass and IMF of meat.

The visual defects ranged from 1.64 to 2.00 points and were not affected by treatment (P>0.10). Also, there were no differences in odour attributes (P>0.10). However, the texture resulted harder and more fibrous in hams from lighter pigs than in those from heavier pigs (P<0.05). RUÍZ-CARRASCAL *et al.* (2002) found that hardness and fibrousness had an inverse relationship with IMF content whereas a significant correlation was detected between IMF or marbling and juiciness. In addition, a lower fibrousness has been also related to lower water losses, due to the different processing length, and also as a consequence of a low salt content and a high proteolitic index (SÁNCHEZ-MOLINERO and ARNAU, 2010). In this sense, a lower salty flavour was detected as pig SW increased (P<0.05) which is in accordance with the differences (trend) found analytically in sodium chloride content. Although high salt content is trying to be avoided in foods for human, this organoleptic attribute is appreciated in products such as dry-cured ham by consumers (CILLA *et al.*, 2005).

Finally, no effect of pig SW was observed on overall quality assessment (P>0.10). It can be explained because the increase of SW would improve some attributes demanded by consumers but also others undesirable. In addition, the lack of a clear effect of pig SW on IMF, whose relation with sensory quality has been well demonstrated, might have also conditioned the final score of panelists.

CONCLUSIONS

The increase of slaughter weight of pigs from 120 to 140 kg (currently accepted by PDO Teruel ham) provided heavier hams that carried out several chemical and sensory differences in the

quality of the final product. If certain homogeneity is desirable, the range of slaughter weight of pigs should be shortened. In addition, future research works in processing technology also would help to reduce some differences, especially in chemical composition.

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PAPER

USE OF TARRAGON (*ARTEMISIA DRACUNCULUS*) ESSENTIAL OIL AS A NATURAL PRESERVATIVE IN BEEF BURGER

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ABSTRACT

Nowdays, adding natural food preservatives is one of the methods for increasing shelf-life. The aim of this study was evaluation of antioxidant activity and antibacterial effects Tarragon (*Artemisia dracunculus*) essential oil (TEO) in beef burger product.

In this experimental study, essential oil of the Tarragon was isolated by hydrodistillation. Then, TEO was analyzed by gas chromatography-flame ionization detector (GC-FID) and gas chromatography/mass spectrometry (GC-MS). The effect of different concentrations of Tarragon essential oil (0.00, 0.062, 0.125, and 0.25%) in 4°±1°C temperature and storage time up to 12 days was evaluated on lipid oxidation, anti *Staphylococcus aureus* activity and organoleptic effects in beef burger.

The monoterpenes hydrocarbons constitute the major fraction of the TEO (95.91%) and the sesquiterpene hydrocarbons were the minor fraction (0.46%). No significant differences were observed after adding of different concentrations of essential oil on lipid oxidation value in raw beef burger ($P>0.05$). The Tarragon essential oil 0.25% in storage temperature (4±1°C) decreased growth rate of *S. aureus* in beef burger ($p<0.05$). Also overall acceptance rate in beef burger containing Tarragon essential oil 0.125% created a better sense in product ($p<0.05$).

Therefore, this essential oil might be used as an antibacterial agent and flavor enhancer in meat products such as beef burger.

- Keywords: antibacterial effect, antioxidant activity, *Artemisia dracunculus*, beef burger -

INTRODUCTION

In recent years, the food industries researchers search for superseded sources of antibacterial and chemical preservatives against inroad of bacteria and lipid oxidation in foods (GUIMARÃES *et al.*, 2010). Meat and its products such as beef burger are widely consumed all over the world. During storage time shelf life of these products is reduced. Oxidation of lipids and degradation of organoleptic agents change the flavor, color and texture of meat products (SALEM *et al.*, 2010). Also, food spoilage and pathogenic bacteria could contaminate meat products and lead to public health hazard and economic losses (SALEM *et al.*, 2010). On the other hand, the use of the chemical antioxidants with high activity, such as TBHQ (tertiary butyl hydroquinone), can threaten human health (RAEISI *et al.*, 2012; KHOSRAVI-BOROUJENI *et al.*, 2012). Natural antioxidants other than possessing protective activities against food spoilage they have therapeutic and protective effects against a wide variety of different diseases (RAFIEIAN-KOPAEI *et al.*, 2013; KHOSRAVI-BOROUJENI *et al.*, 2012). The presence of limonene, carvacrol and eugenol as bioactive substances in essential oils may exert inhibitory effect on microorganisms growth and reduce deterioration due to lipid oxidation in foods (SENGUL *et al.*, 2011; GUIMARÃES *et al.*, 2010).

The *Artemisia dracunculus* is commonly consumed in the most food as smell and flavor enhancer in barbecues, salads and soup. The *Artemisia dracunculus* is a small shrub from the *Asteraceae* family and is called "Tarkhon" in Iran, Tarragon, dragon wormwood, dragon sagewort, estragon. In traditional medicine its used for the treatment of collywobbles, fever, diabetes and bacterial or parasitic infections (RAEISI *et al.*, 2012; AYOUGHI *et al.*, 2011). Previous studies have shown the antioxidant and antibacterial activities of essential oil of *Artemisia dracunculus* on kind of bacteria in vitro (EREL *et al.*, 2012; AYOUGHI *et al.*, 2011; SENGUL *et al.*, 2011; KORDALI *et al.*, 2005).

The aim of this study was evaluation of antioxidant and antibacterial effects of Tarragon (*Artemisia dracunculus* L.) essential oil in beef burger.

MATERIALS AND METHODS

Preparation of the essential oil

The Tarragon was purchased from the local grocery of Shahrekord city and identified by the standard botanic work in Medical Plants Research Center in Shahrekord University of Medical Sciences, Iran. The essential oil of Tarragon leaves (TEO) was extracted by steam distillation (1:5 herb/water, in w/v ratio) for 3 h using a Clevenger type apparatus and dried by adding anhydrous sodium sulphate as well as stored at 4°C before being used for assay (RAEISI *et al.*, 2012).

Analysis of essential oil

The essential oil was analyzed using a Younglin Acme 6000 GC-FID with a HP-5MS capillary column (30 m × 0.25 mm, film thickness 0.25 μm). Helium as carrier gas was used at a flow rate of 0.8 ml/min. The essential oil was diluted in n-pentane (1/1000, v/v) and 1.0 μL injected in the splitless mode. The primary oven temperature was maintained at 50°C for 5 min and then increased to 240°C at the rate of 3°C/min. Temperatures of injector and detector were 290° and 300°C, respectively. Quantitative data were obtained from GC peaks area percent.

Then GC/MS essential oil analysis was performed on a HP-5MS capillary column (30 m × 0.25 mm, film thickness 0.25 μm), using a Gas Chromatograph Agilent 6890, interfaced with a Mass Spectrometer Agilent HP- 5973. The electron ionization (EI) system with ionization energy of 70 eV and temperature of ion source 220°C was used for GC–MS detection. Other stages were under similar conditions as GC. Mass spectra were scanned between 50 and 550 a.m.u range. The essential oil compounds were identified by retention indices (RI) and compared their RI with data reported in the articles, references books as well as standard libraries (Wiley275.L and Wiley7n.L) (ADAMS, 1995).

Preparation of beef burger

Five kg beef burger was taken from a batch production of a meat products factory. For microbiological analysis, samples (100 g) of beef burgers were placed in the stomacher bags and transported to the Atomic Energy Organization of Tehran, Iran for sterilization with Gamma irradiation (60 Cobalt emitting gamma rays, period time 26 minutes for 5.5 KGy). Also microbial culture was carried out for confirmation of any growth bacteria. The samples were divided into treated and untreated samples (control). The treated groups were added 0.062, 0.125 and 0.25% concentrations of Tarragon essential oil. All samples were labeled and stored at 4 °C. Then, the samples were analyzed on days 0, 1, 3, 6, 9 and 12 for chemical and microbiological factors (NOORI *et al.*, 2012).

Determination of lipid oxidation

Thiobarbituric acid (TBA) assay was conducted as described by MARASCHIELLO *et al.* (1999) with some modifications. 0.5 g of raw beef burger samples was added to 10 mL of deionized water and mixed vigorously for 1 min and then 2.5 mL of 25 % TCA (Trichloroacetic acid) (Sigma-Aldrich Corporation, St. Louis, MO., USA) was added. The samples were stored for 15 min at 4 °C

Table 1 - Composition of Tarragon essential oil.

Name	Consentration (%)
α-pinene	0.57
β-pinene	0.11
β-myrcene	0.10
Limonene	1.79
z-β-ocimene	3.42
Trans-ocimene	3.86
Terpinene	0.08
Linalool	0.16
Ocimene (allo)	0.16
Methyl chavicol	84.83
Geranial	0.17
Iso bornyl acetate	0.10
Eugenol	0.12
Iso safrole (E)	0.37
Methyl eugenol	0.07
Valencene	0.1
β-sesquiphellandrene	0.07
Cinnamaldehyde (para-methoxy)	0.20
Spathulenol	0.09
Monoterpenes	10.09
Oxygenated monoterpenes	85.82
Sesquiterpenes	0.17
Oxygenated sesquiterpenes	0.29
Total	**96.37**

and centrifuged for 5 min (4000 rpm, at 4 °C). The 3.5 mL of supernatant was mixed with 1.5 mL of 0.6 % TBA (Sigma-Aldrich Corporation, St. Louis, MO., USA) and placed in water bath (70 °C) for 30 min. The absorbance of the solutions was measured at 532 nm with spectrophotometer (Unico UV-2100, USA) against a blank containing of 2.5 mL of deionized water, 1 mL 25% TCA and 1.5 mL 0.6% TBA. The standard curve was prepared by standard solutions of 1, 1, 3, 3-tetraethoxypropane (TEP) (Sigma-Aldrich Corporation, St. Louis, MO., USA). The amount of TBA was expressed as mg of malonaldehyde (MDA) per kg of meat.

Preparation of inocula and enumeration of *S. aureus*

Staphylococcus aureus with PTCC 1189 (Persian Type Culture Collection) was obtained from the Iranian Research Organization for Science and Technology (IROST), Iran. The lyophilized bacterium was moved to Brain heart infusion (BHI) (Merck Ink. Darmstadt, Germany) and was incubated at 37°C for 18 h.

For the test, final inoculum of pathogen cells of 10^3 colony forming unit (CFU)/g of *S. aureus* to beef burgers samples were used by spectrophotometer (absorbance 600 nm) and surface cultivation, simultaneously (SHEKARFOROUSH *et al.*, 2007).

Sensory evaluation

A hedonic test was used for sensory evaluation described by AMANY *et al.* (2012). Cooked samples were served warm to 6 members of household trained panel without care of age or sex. A nine-point hedonic scoring scale (1 =dislike extremely, 2= dislike very much, 3= dislike moderately, 4= dislike slightly, 5= neither like nor dislike, 6= like slightly, 7= like moderately, 8, like very much, 9= like extremely) was used for overall acceptability.

Statistical analysis

The data concerning this present study were framed as means±standard deviation of triplicates. The significance of difference was performed by Kruskal- Wallis test and Friedman test using INSTAT software. $P<0.05$ was considered to be significant.

RESULTS

The composition of the TEO is demonstrated in Table 1. Nineteen compounds were identified in the *Artemisia dracunculus* essential oil samples, representing the 96.37% of the total oil. The main compound of TEO were methyl chavicol (84.83%), trans-ocimene (3.86%) and z-β-ocimene (3.42%). The monoterpenes hydrocarbons constitute the major fraction of the oil (95.91%) and Sesquiterpene hydrocarbons amounted to 0.46%.

Table 2 shows the mean TBA values for raw beef burgers with different concentrations of

Table 2 - Mean TBA values (mg of malonaldehyde/kg) for raw beef burger with different concentration of Tarragon essential oil (TEO) during refrigerated storage (4°±1°C) for 12 days.

Days Treatment	0	1	3	6	9	12
Control	0.95±0.02^a	1.04±0.04^a	1.36±0.01^a	1.92±0.02^a	2.57±0.02^a	3.22±0.02^a
BHT 0.25%	0.95±0.02^a	0.97±0.01^a	1.12±0.02^b	1.51±0.02^b	2.14±0.01^b	2.86±0.01^b
TEO 0.0625%	0.95±0.02^a	0.97±0.01^a	1.35±0.01^a	1.91±0.01^a	2.54±0.01^a	3.22±0.01^a
TEO 0.125%	0.95±0.02^a	0.95±0.01^b	1.33±0.02^a	1.86±0.01^a	2.49±0.01^a	3.18±0.01^a
TEO 0.25%	0.95±0.02^a	0.95±0.01^b	1.28±0.02^a	1.76±0.01^a	2.23±0.01^a	3.13±0.01^a

The different superscripts within the same column are significantly different ($p < 0.05$).

Table 3 - Effect of different concentration of Tarragon essential oil (TEO) on *Staphylococcus aureus* in raw beef burger during refrigerated storage (4°±1°C) for 12 days.

Days Treatment	0	1	3	6	9	12
Control	$1.5\times10^3\pm48^a$	$1.2\times10^3\pm57^a$	$1.7\times10^3\pm57^a$	$<100^a$	$<100^a$	$<100^a$
TEO 0.0625%	$1.5\times10^3\pm48^a$	$5.3\times10^2\pm152^a$	$<100^b$	$<100^a$	$<100^a$	$<100^a$
TEO 0.125%	$1.5\times10^3\,48^a$	$1\times10^2\pm1^a$	$<100^b$	$<100^a$	$<100^a$	$<100^a$
TEO 0.25%	$1.5\times10^3\,48^a$	$<100^b$	$<100^b$	$<100^a$	$<100^a$	$<100^a$

The different superscripts within the same column are significantly different ($p < 0.05$).

Tarragon essential oil (TEO) during refrigerated storage (4±1°C) for 12 days. Control sample showed the highest TBA values and in the TEO 0.25% treatment after day 1 lower than the control in both raw beef burgers ($p<0.05$). On other days BHT 0.25% showed lower oxidation values than control group ($p<0.05$) and no statistically significant differences ($p>0.05$) were observed between the TBA values TEO treatment samples and the control sample.

The time-related survival of *S. aureus* following treatment with different concentrations of TEO is demonstrated in Table 3. The Tarragon essential oil in storage temperature (4±1°C) decreased growth rate of *S. aureus* till third storage day in beef burger than control group ($p<0.05$).

Fig. 1 shows overall acceptability values for all cooked beef burgers treated to TEO up to 12 days. The results of organoleptic evaluation demonstrated that the best overall acceptability related to 0.125% TEO treated samples ($p<0.05$).

Furthermore, after third day of storage all sensory attributes were declined for control samples while TEO treated samples were scored between 5.5 and 7.5. After sixth day of storage all of the samples revealed reduction of overall acceptability values.

DISCUSSION

It is obvious that different plant essential oils have antioxidant and antibacterial compounds (RAEISI *et al.*, 2012). On based results obtained in this study, essential oil of Tarragon had antioxidant and antibacterial properties. Nineteen compounds were identified, accounting for 96.36% of the essential oil for *Artemisia dracunculus*. The main constituents of essential oil were Methyl chavicol (84.83%), Trans-ocimene (3.86%), z-β-ocimene (3.42%), Limonene (1.79%) and α-pinene (0.57%). In previous studies *Artemisia dracunculus* essential oil contained (Z)-anethole (81.0%), z-β-ocimene (6.5%), (E)-β-ocimene (3.1%), limonene (3.1%) from Turkey (KORDALI *et al.*, 2005), and (*Z*)-anethole (51.72%), z-β-ocimene (8.32%), methyleugenol (8.06%) from Iran (AYOUGHI *et al.*, 2012). There is some similarity between the compound of TEO of this study and the above studies. The

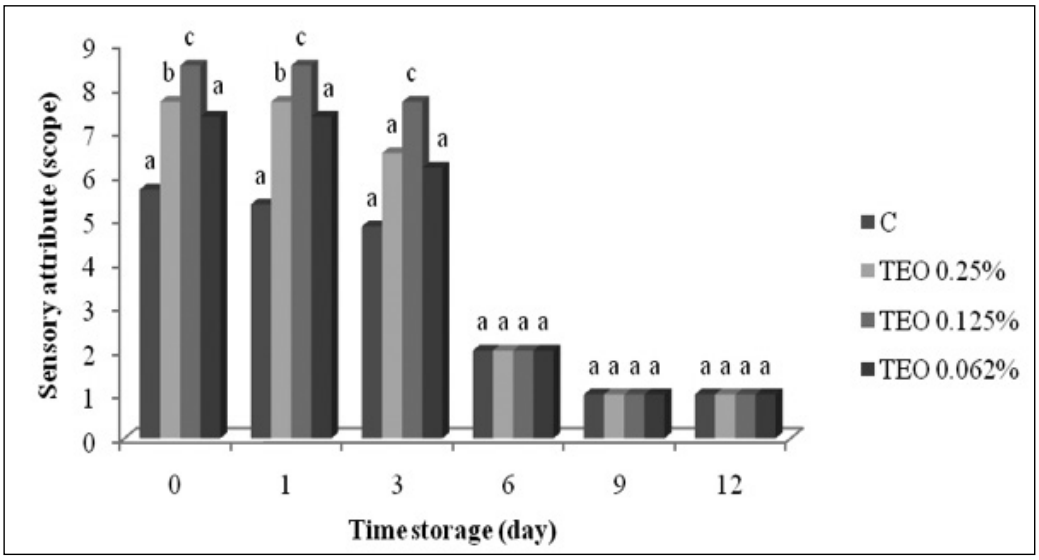


Fig. 1 - Overall acceptability evaluation of cooked beef burger mixed with different concentration of Tarragon essential oil (TEO) (Different letters have significant difference ($p < 0.05$).

some variations chemical compositions might be related to different locations of sampling. Also, weather conditions can change year to year in essential oil compound.

There was seen in Table 1 that lipid oxidation increased over time in all samples. Increase of TBA values may be caused auto-oxidation of meat lipids, bacteriological and oxidative rancidity (SALEM *et al.*, 2010). Nevertheless, the antioxidant activity of TEO were significant in 0.25 and 0.125% concentration of essential oil in first storage day of beef burgers ($p<0.05$) but in other days up to twelfth day was not observed significant difference in all of TEO treatment samples with control group ($p>0.05$). These low antioxidant effects of TEO can be caused by low levels of phenolic and flavonoid compounds. Although, Previous studies are demonstrated the antioxidant activity of medicinal plants and spice essential oils such as *Artemisia dracunculus in vitro* (AYOUGHI *et al.*, 2011; JAZANI *et al.*, 2011; SHARAFATI CHALESHTORI *et al.*, 2011). Activity of EOs is due to their composition as phenolic and flavonoid compounds that can donate hydrogen in oxidation reactions, scavenge free radicals and chelate to metallic ions (VIUDA-MARTOS *et al.*, 2010).

Staphylococcus aureus is a food borne pathogen and can cause a transmissible disease by inappropriate handling and storage of food contaminated with staphylococci as well as in many countries it is as the third most pathogen responsible for outbreaks of food poisoning (DE SOUZA *et al.*, 2009). These results showed that TEO has an antibacterial activity against *S. aureus.* The counted number of bacteria showed a decrease in the count of *S. aureus* with increasing essential oil concentration. Indeed, 0.25% TEO and 0.125% TEO in beef burgers after 24 hours reduced the viable cell counts by a 2 log CFU/g at 4°C though the number of bacteria in the control was approximately constant (3 log CFU/g). After six days of storage, the control samples revealed decreasing on *S. aureus* counts. The reason for the decrease in the counts of *S. aureus* may be due to the sensitive of this bacterium against temperature of refrigerator (4°±1°C). Previous works have shown similar results about effects of medicinal plant extracts and essential oils on *S. aureus* in several food models (JAGADEESH BABU *et al.*, 2012; CHOOBKAR *et al.*, 2010) MAHDAVIAN MEHR *et al.* (2010) evaluated Nowroozak leaf extract on growth of *S. aureus* in hamburger and then stored at -12°C. Its results demonstrated that the number of *S. aureus* in all samples with different concentrations of extract reduced during storage. The presence of antibacterial compound as polyphenols and carvacrol in essential oil of plants is more effective on the gram-positive bacteria than gram-negative bacteria due to their influence on membrane fluidity (MAHDAVIAN MEHR *et al.*, 2010: ROMANO *et al.*, 2009). The results obtained in this study are also consistent with above studies.

The results showed that the best overall acceptability related to 0.125% TEO. The significant improvement of TEO treated samples compared to control samples is due to addition of Tarragon essential oil and this could be related to its aromatic compounds (KASSEM *et al.*, 2011). However, at the used TEO 0.25% exhibited more antibacterial activity against *S. aureus*, its addition in beef burgers had lower overall acceptability than 0.125% TEO. Therefore, high concentration of essential oils, their practical consume is limited due to a negative smell–taste effect in foods. We are suggested that TEO may be used as a part of combination with other preservation systems to decrease the amount of antimicrobial required and unfavorable sensorial effects (HAYOUNI *et al.*, 2008).

CONCLUSIONS

With mentioned to the dose-related antibacterial activity of Tarragon essential oil in this work is suggested that TEO can be used as a food natural preservative and food flavouring in beef burger products and meat industries.

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PAPER

NUMERICAL ANALYSIS OF FLUID FLOW VELOCITY IN SELECTED ELEMENTS OF PIPELINE INSTALLATIONS WITH REFERENCE TO THE EFFECTS OF CLEANING

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ABSTRACT

This paper presents the results of research on the use of commercial computer applications in fluid mechanics calculations. Such applications are viewed as tools enabling the identification of problems related to the maintenance of cleanliness in closed industrial installations which may occur due to unfavorable flow conditions. By using CFD (Computer Fluid Dynamic) methods in selected elements of pipe installations, it was possible to show the distributions of pressure, velocity and shear stress on the walls and to analyze these in terms of the CIP system (cleaning in place). The results were verified by experimental research performed in a CIP cleaning station which assessed the cleanliness of the tested pipe elements after the cleaning process. The outcomes of the research are promising and prove the appropriacy of using CFD methods in predicting and modeling the hygienic efficacy of food industry equipment.

- Keywords: CIP, wall shear stress, CFD, elbow, tee, valve -

INTRODUCTION

Hygiene is one of the most important elements in food processing. It is defined as all measures and actions ensuring that food production is safe for the consumer, considering its intended use (Regulation (EC) No 852/2004, Regulation (EC) No 853/2004). Among the most important actions within the scope of the principles of GHP (Good Hygiene Practice) are the cleaning and disinfection processes governing production facilities featuring various devices and small ancillary equipment. These processes are carried out on the basis of different techniques, the choice of which is highly dependent on the accessibility of the surfaces to be cleaned and on the design of equipment and apparatuses. Cleaning in the CIP system is a highly automated process which does not require disassembly of the items to be cleaned - suitable cleaning solutions are passed through the installation in question. During their use, cleaning solutions cause the wetting and disintegration of post-production pollution.

All equipment and production lines involved in the production of liquid or semi-liquid foods undergo CIP cleaning. These pieces of equipment can be divided into three groups: flow apparatuses characterized by a complex structure (e.g. centrifuges, whippers, filters, funnels, heat exchangers); industrial vessels (reservoirs, tanks, tubs, chambers, cisterns); and pipelines, which are an essential part of the production installation and transport liquid raw materials and semi-finished products between successive components involved in its manufacture. Pipelines and their components are present in each production installation. Typical elements of piping systems are elbows, tees, couplings, gaskets, and valves. Due to the cleaning conditions, the best and safest solution is to use only straight sections of pipe (EHEDG, 2001; UNI EN 1672-2, 2009; DIAKUN, 2013). However, the design of the equipment often requires a number of curvatures (elbows), tees, blind ends and extra valves (LELIEVRE *et al.*, 2002b; JENSEN *et al.*, 2005). Such elements are more difficult to clean than the straight sections of pipes and exert a negative impact upon hygiene maintenance.

The effectiveness of a CIP cleaning system depends on four factors: mechanical energy causing the physical break-down of sediments from the surface being cleaned, the chemical energy boosting break-down of impurities from the surface by emulsifying the fat, protein peptizing, dissolution and dispersion of mineral impurities in the cleaning solution, and their drainage from the system. Thermal energy intensifies the two previous factors and the cleaning time, meaning the contact of various cleaning solutions of different intensity and different temperatures with contaminants (BREMER et. al 2006; BLEL *et al.*, 2007; PIEPIÓRKA, 2008). Hydrodynamic parameters in the flow cleaning process are the most important factors in the removal of post-production sediments (LELIEVRE *et al.*, 2002a; JENSEN and FRIIS, 2007). Many researchers have shown that the kinetics of fouling removal is a function of the flow rate of cleaning detergents, the Reynolds number (1) and shear stress forming on the walls of the elements to be cleaned (2) (JENSEN *et al.*, 2005; BLEL *et al.*, 2007; DIAKUN *et al.*, 2010), where shear stress τ_w for Newtonian fluid is defined as the product of a dynamic viscosity μ and flow velocity gradient $\partial u/\partial y$ at a given distance from the wall element y (2).

$$\mathrm{Re} = \frac{\rho \cdot u \cdot d}{\mu} \quad (1)$$

$$\tau_w = \mu\left(\frac{\partial u}{\partial y}\right) \quad (2)$$

Elbows, throats, branches or valves cause a disruption of the fluid and the pressure drops due to local resistance and friction (KUSUMANINGRUM *et al.*, 2003; LELIEVRE *et al.*, 2002a). In these elements, there may be some areas in which the flow of the fluid is very slow or even stagnant. The closed nature of the structure and the lack of access to the surfaces to be cleaned cause difficulties in monitoring these places and assessing their cleanliness. One way, among others, to determine the conditions of flow in closed industrial installations is to use numerical analysis based on the CFD code. This tool is also widely used by many researchers not only to assess the cleaning conditions in CIP systems (LELIEVRE *et al.*, 2002b; JENSEN *et al.*, 2005; JENSEN and FRIIS, 2007; RAHAMAN *et al.*, 2008; PIEPIÓRKA-STEPUK and JAKUBOWSKI, 2013; KUBIAK and JAKUBOWSKI 2013).

THE AIM OF THE RESEARCH

The aim of the research was to determine the conditions of the 3D flow in selected elements of pipelines, i.e. elbows, tees, blind ends, and valves (butterfly and ball), by using numerical calculations based on CFD code. Based on numerical analysis, the distribution of the velocity field, wall shear stress and streamlines in pipeline spaces was ascertained. The results of the research determined those places in pipes which are difficult to clean in the flow. Using the numerical results, areas were identified where low velocity occurs in elements of pipes. These formed the basis for selecting areas to assess the cleanliness of particular areas of the elements of pipes which were cleaned in the real conditions in a laboratory CIP cleaning station. The results provided information about the dependency between the form of the pipe and the efficiency of the cleaning operation.

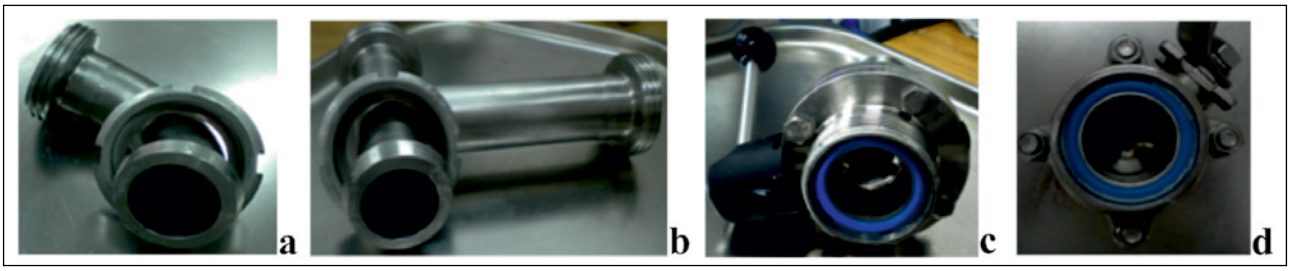


Fig. 1 - Pipeline elements undergoing the process of soiling and cleaning: a - elbow; b – a dead end; c – butterfly valve; d – ball valve.

MATERIALS AND METHODS

The study was conducted in two stages. In the first stage, a geometric model was developed for numerical computations. Flow conditions were modeled in selected elements of the pipelines (Fig. 1). On the basis of the velocity flow and wall shear stress distributions obtained via numerical analysis, the areas in the above-mentioned elements were identified, in which potentially unfavorable conditions for cleaning in the flow occurred. In the second stage of the study, cleaning in the laboratorial CIP cleaning station was carried out, assessing the degree of cleanliness of the indicated areas.

Numerical simulations

Computations were performed with the finite volume method (FVM) on which the ANSYS CFX program, one of the modules for numerical computation, is based. CFX constitutes a set of tools and elements for computation, as well as analysis of fluid flow and accompanying phenomena. The program has a wide range of possibilities for declaring boundary conditions and material properties (ANSYS, 2009). The results of our computations are shown graphically as streamlines of velocities, pressure distributions and shear stresses on the walls for selected elements of the pipelines. Computations were conducted for elbows, tees, dead ends and two kinds of valves: ball and butterfly. The characteristic size of the pipe elements is depicted in Table 1.

Table 1 - Characteristic dimension of the examined elements.

Characteristic dimension	Value
Internal diameter of the tested elements [m]	d = 0.038
Wall thickness [mm]	b = 1.2
Cross sectional area [m^2]	A = 0.00113
Calculated average of flow velocity [m×s^{-1}]	u = 1.5

Each of the analyzed models was meshed using a quadratic regular grid with an element size of 0.001 m (Fig. 2). The mesh was created as shown by JENSEN and FRIIS (2007). As a result, the numbers of cells in each were more than 500 000.

In order to perform numerical calculations, isothermal flow conditions were assumed. The fluid corresponded to the water with viscosity and density at temperature of T = 45°C. The flow conditions were established as time-invariant and the flow was described as stable. It was also assumed that the fluid flow is turbulent and the turbulence models include the standard definition of the description of the boundary layer. Thus, the κ - ε model was used for calculations, where flow is described by two equation turbulence models in the near-wall cell where dissipation is algebraically fixed (JENSEN and FRI-

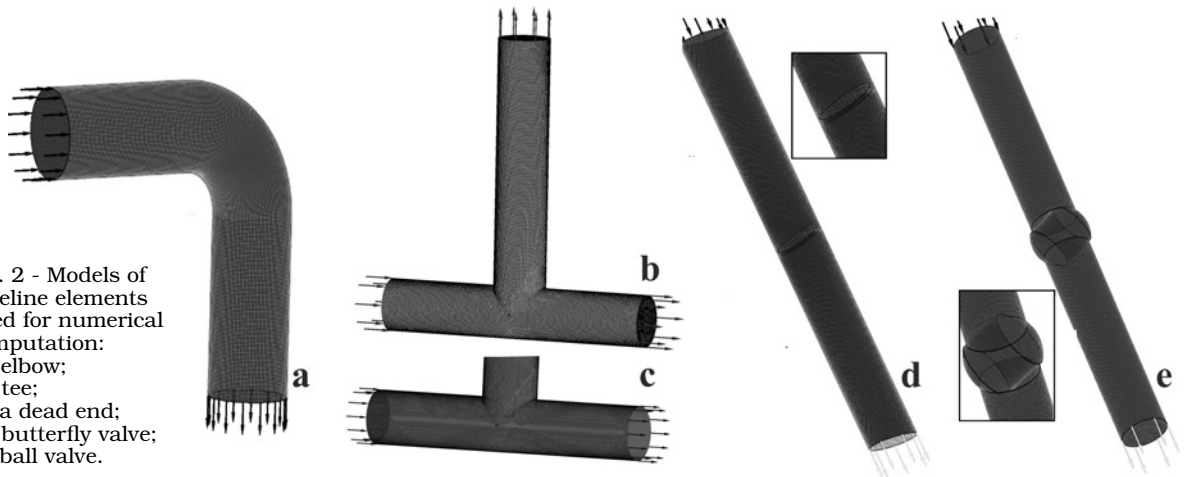


Fig. 2 - Models of pipeline elements used for numerical computation:
a - elbow;
b - tee;
c - a dead end;
d - butterfly valve;
e - ball valve.

IS, 2007). Boundary and initial conditions at the first step of the first iteration were defined as: zero velocity value on the walls (3), zero value of turbulent kinetic energy coefficients κ (4) and the dissipation of turbulence ε (5), mass flow rate at the inlet and the outlet of the numerical tested models at $\dot{m}$ = 1.95 kg×s^{-1} level. The flow rate was adopted based on the preliminary test measurements of the flow velocity in the real channel pipe, for which the average flow velocity was u = 1.5 m×s^{-1}. Surface roughness was assumed at the level R_a = 0.4 μm (EHEDG, 2007).

$$u_x = 0; u_y = 0; u_z = 0 \tag{3}$$

$$\rho u_x \frac{\partial \kappa}{\partial x} + \rho u_y \frac{\partial \kappa}{\partial y} + \rho u_z \frac{\partial \kappa}{\partial z} = 0 \tag{4}$$

$$\rho u_x \frac{\partial \varepsilon}{\partial x} + \rho u_y \frac{\partial \varepsilon}{\partial y} + \rho u_z \frac{\partial \varepsilon}{\partial z} = 0 \tag{5}$$

Model formulation

The models are described in a rectangular coordinate system x, y, z (in the Cartesian coordinate system). Apart from the components of the gravity force and assuming, according to the Reynolds hypothesis, that the instantaneous values of all the physical quantities characterizing the flow can be considered as the sum of the averaged time values, an average flow can be described using equations of the conservation of mass (6) and momentum (7).

Bernoulli's equation in divergence form

$$\frac{\partial \rho}{\partial t} + \rho \, div \, \vec{u} = 0 \tag{6}$$

Momentum balance equation (Navier - Stokes) as a vector for an incompressible fluid has the following form:

$$\begin{cases} u_x \frac{\partial u_x}{\partial x} + u_y \frac{\partial u_x}{\partial y} + u_z \frac{\partial u_x}{\partial z} = -\frac{1}{\rho}\frac{\partial p}{\partial x} + \nu\left(\frac{\partial^2 u_x}{\partial x^2} + \frac{\partial^2 u_x}{\partial y^2} + \frac{\partial^2 u_x}{\partial z^2}\right), \\ u_x \frac{\partial u_y}{\partial x} + u_y \frac{\partial u_y}{\partial y} + u_z \frac{\partial u_y}{\partial z} = -\frac{1}{\rho}\frac{\partial p}{\partial y} + \nu\left(\frac{\partial^2 u_y}{\partial x^2} + \frac{\partial^2 u_y}{\partial y^2} + \frac{\partial^2 u_y}{\partial z^2}\right), \\ u_x \frac{\partial u_z}{\partial x} + u_y \frac{\partial u_z}{\partial y} + u_z \frac{\partial u_z}{\partial z} = -\frac{1}{\rho}\frac{\partial p}{\partial z} + \nu\left(\frac{\partial^2 u_z}{\partial x^2} + \frac{\partial^2 u_z}{\partial y^2} + \frac{\partial^2 u_z}{\partial z^2}\right). \end{cases} \tag{7}$$

The turbulence equation

The equation for kinetic energy κ

$$\rho u_x \frac{\partial \kappa}{\partial x} + \rho u_y \frac{\partial \kappa}{\partial y} + \rho u_z \frac{\partial \kappa}{\partial z} = \frac{\partial}{\partial x}\left(\frac{\mu_e}{\sigma_\kappa}\frac{\partial \kappa}{\partial x}\right) + \\ + \frac{\partial}{\partial y}\left(\frac{\mu_e}{\sigma_\kappa}\frac{\partial \kappa}{\partial y}\right) + \frac{\partial}{\partial z}\left(\frac{\mu_e}{\sigma_\kappa}\frac{\partial \kappa}{\partial z}\right) + G - \rho\varepsilon \tag{8}$$

The equation for the dissipation rate ε

$$\rho u_x \frac{\partial \varepsilon}{\partial x} + \rho u_y \frac{\partial \varepsilon}{\partial y} + \rho u_z \frac{\partial \varepsilon}{\partial z} = \frac{\partial}{\partial x}\left(\frac{\mu_e}{\sigma_\varepsilon}\frac{\partial \varepsilon}{\partial x}\right) + \\ + \frac{\partial}{\partial y}\left(\frac{\mu_e}{\sigma_\varepsilon}\frac{\partial \varepsilon}{\partial y}\right) + \frac{\partial}{\partial z}\left(\frac{\mu_e}{\sigma_\varepsilon}\frac{\partial \varepsilon}{\partial z}\right) + C_1\frac{\varepsilon}{\kappa}G - C_2\rho\frac{\varepsilon^2}{\kappa} \tag{9}$$

In the above equations the following factors appear: energy dissipation for averaged motion,

$$G = \mu_t\left\{2\left[\left(\frac{\partial v_x}{\partial x}\right)^2 + \left(\frac{\partial v_y}{\partial y}\right)^2 + \left(\frac{\partial v_z}{\partial z}\right)^2\right] + \left(\frac{\partial v_x}{\partial x} + \frac{\partial v_y}{\partial y} + \frac{\partial v_z}{\partial z}\right)\right\} \tag{10}$$

$$\mu_t = \rho C_\mu \frac{\kappa^2}{\varepsilon} \tag{11}$$

effective viscosity, as a sum of the dynamic viscosity and turbulent viscosity determined by the equation,

$$\mu_e = \mu + \mu_t \tag{12}$$

and the constant turbulence model C_μ = 0.09 and the values of the coefficients σ_κ = 1.0;

$$\sigma_\varepsilon = 1.3; \; C_1 = 1.44; \; C_2 = 1.92$$

Mass conservation equations (6), the equation of the conservation of momentum (7) and the equation turbulence model adopted κ - ε (8, 9) are the mathematical models of the motion of a fluid in the channel of a pipeline. These models are partial differential equations, in which the independent variables are the coordinates of the point considered in the x_i. On this basis, it is possible to determine the distribution of the dependent variables, such as the components of the velocity vector u, the pressure p, turbulent kinetic energy κ and the dissipation of turbulence ε (PIEPIÓRKA-STEPUK and DIAKUN, 2014; JEONG and SEONG 2014).

Based on the numerical results, zones are determined in selected elements of the pipeline where the flow conditions can be disadvantageous to cleaning in flow.

Laboratory experiments

In experimental studies conducted in a laboratory CIP cleaning station, the internal surfaces of the elements were soiled with milk, according to the method presented by PIEPIÓRKA-STEPUK and DIAKUN in 2014. This resulted in a difficult to remove whey protein – fat fouling layers. Then, the contaminated elements were installed in a pipeline and cleaned with clean water, flowing at a velocity of u = 1.5 m×s^{-1}. The chemical factor was deliberate-

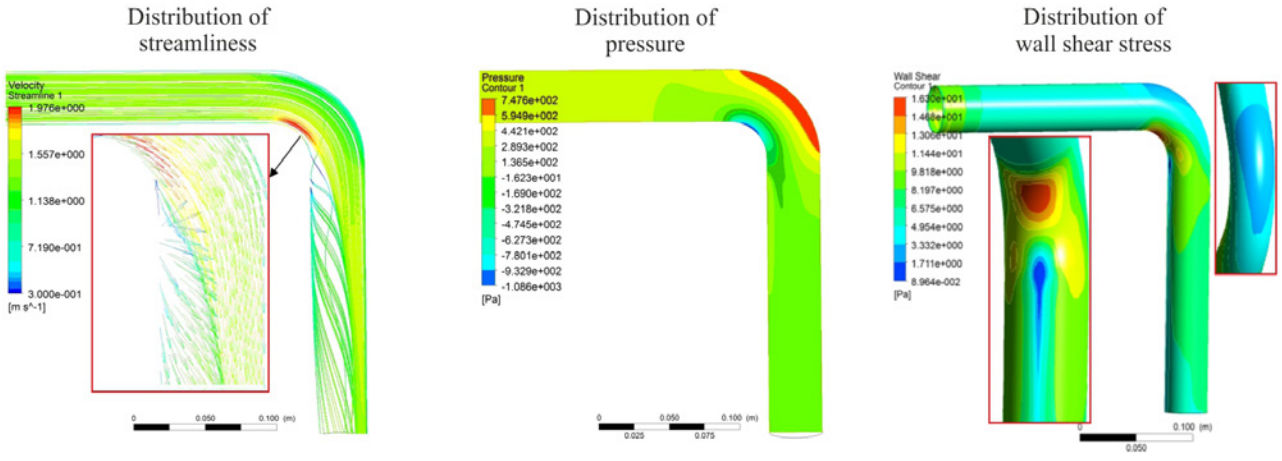


Fig. 3 - The results of numerical investigations on the elbow pipelines.

ly eliminated from cleaning programs, thus allowing the research to realize its objectives relating solely to the evaluation of hydrodynamic cleaning factors. The cleaning time *t*, as one of the factors determining a clean surface, was set at 3; 5; 10; 20 and 40 minutes. The water temperature was set at a constant level of *T* = 45°C, which results from the studies by DIAKUN (2011a) and CHEN *et al.* (2003). These studies showed that protein-fat sediments derived from milk are most effectively removed at this temperature. The final program of the experimental research consisted of five cleaning programs. The cleanliness of the surface was assessed by Clean -Trace™ Surface Protein Plus tests (DIAKUN, 2011b). Cleaning efficiency was expressed on a scale from 0 to 10, where 0 is the initial state of contamination, and 10 a surface completely clean. Numerical simulations made it possible to identify areas of reduced flow velocities in the tested elements. Thanks to these calculations based on hydro-mechanical flow interactions on the walls of an item being cleaned, it was possible to identify zones in which favorable and unfavorable conditions for cleaning may occur. The sampling areas in experiments determined in the results of numerical analysis shown in Figures 4, 8, 11, 13.

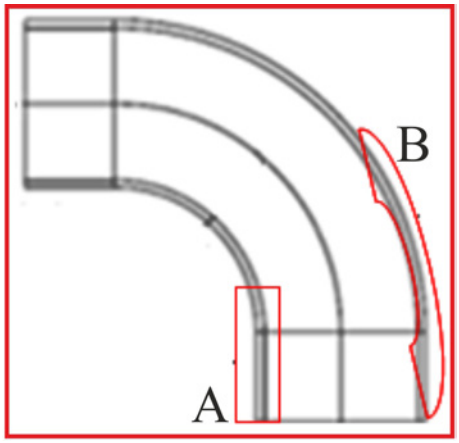


Fig. 4 - Selected sampling areas adopted for experimental research: A - the surface of the pipe below the internal curve; B - the area on the outer arc.

RESULTS AND DISCUSSION

Analyses for each element of the pipes are presented in the following order. The first part presents the results of numerical analysis in the form of streamlines, pressure and shear stress. On this basis, zones were determined which are difficult to clean in a flow. Then, the results of experimental research into the degree of cleanliness of specified areas are presented.

Pipeline elbows

The conducted numerical analyses indicate that the largest flow velocity and a large velocity gradient are present at the inner radius of the elbow (Fig. 3). The lowest flows occur below the curve and on the outer radius. The analysis of velocity vectors and streamlines leads to the conclusion that the most unfavorable area is the pipe surface below the inner curve. The separation of fluid stream occurs here, as well as the changes in flow direction and a turbulence unfavorable to cleaning processes. Flow velocity in these areas decreases to a value close to 0 $m \times s^{-1}$, the shear stresses also drop - their value is ≈ 0.009 Pa. Pressure distribution in the examined model indicates that the greatest pressures occur on the outer edge of the elbow and decrease while approaching the inner edge of the elbow. Pressures should be read as increments for the reference pressure of P_r = 1 atm. Based on the results of numerical analyses, two areas with difficult cleaning conditions were identified in the elbow; these are the area below the

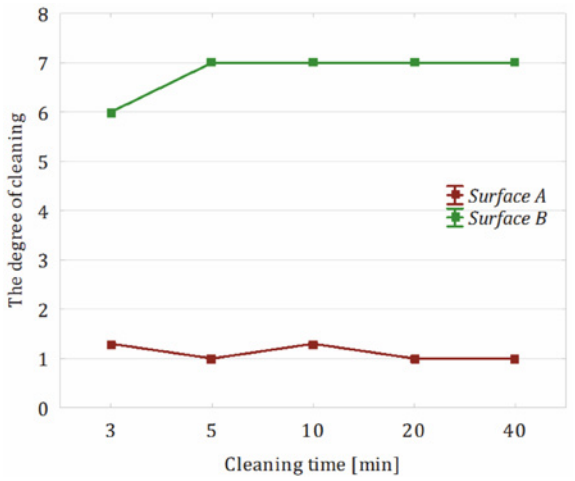


Fig. 5 - Each of the points represents the experimental research into the effectiveness of cleaning the elbow: A - the surface of the pipe below the inner curve; B - the area of the pipe on the outer arc.

inner arc of the elbow and the area on the outer arc (Fig. 4). In Figure 5, each point represents the experimental arithmetic mean of three measurement repetitions concerning cleaning performance for the indicated areas.

The results of experimental research indicate a clear difference between the effectiveness of cleaning surfaces on the inner arc and the effectiveness of cleaning the outer arc. The effectiveness of cleaning the surface of the outer arc at the entrance stood at 6 - 8 purity points. The area below the inner arc of the elbow, right at the outlet achieved 1 and 1.3 cleanliness points. Despite the low flow velocity, the arc on the outer surface was appropriately cleaned. This means that, besides the flow velocity, high pressure has an influence on the cleaning results via the fluid impingement into the wall, whereas a low degree of cleaning on the inner arc is caused by both low (zero) flow velocity and low pressure. Even a longer time could not have improved the final effects of cleaning. In relation to other pipeline surfaces, there were far more impurities in this place than in other areas. Laboratory results, therefore, confirm the results of numerical analyses, where unfavorable flow conditions for the cleaning process were indicated in these areas.

Tees and dead ends

The results of numerical calculations for tees show that the largest flow rate is obtained on the outer wall (B), located perpendicularly to the fluid flow direction (Fig. 6). A rapid change in the directional flow of the flowing liquid causes unfavorable turbulence on the wall of the tee at the inflow (A). At this point, due to reduced flow velocity ($u \approx 0.4$ m×s^{-1}), residual layers of fouling and adverse cleaning conditions can be expected. Reduced flow velocity in the analyzed tee also occurs at the outlets of the pipeline (C). From an initial value of $u = 1.5$ m×s^{-1}, the flow velocity drops to approximately $u = 0.1 \div 0.3$ m×s^{-1}. This process occurs due to liquid separation. The fluid turbulences are not present here and the basic condition for the cleaning process in the flow, which is turbulent motion, is fulfilled. For the flow velocity at the outlet of the Reynolds number is obtains $Re \approx 6000 \div 19\,000$ and therefore the flow has a turbulent character. In this area low wall shear stress occurs, so unfavorable conditions for cleaning could be created here.

In the case of dead ends, the turmoil of the liquid occurs as a result of the inflow in an empty zone (Fig. 7). Numerical analyses show that in long dead end pipelines one can also observe stagnant phenomena. The decrease in media flow velocity in the pockets causes both product and cleaning media retention, particularly if they are directed downwards. Therefore, such solutions should be particularly avoided. However, in tees directed upwards one should expect difficult access for cleaning agents. The sediments

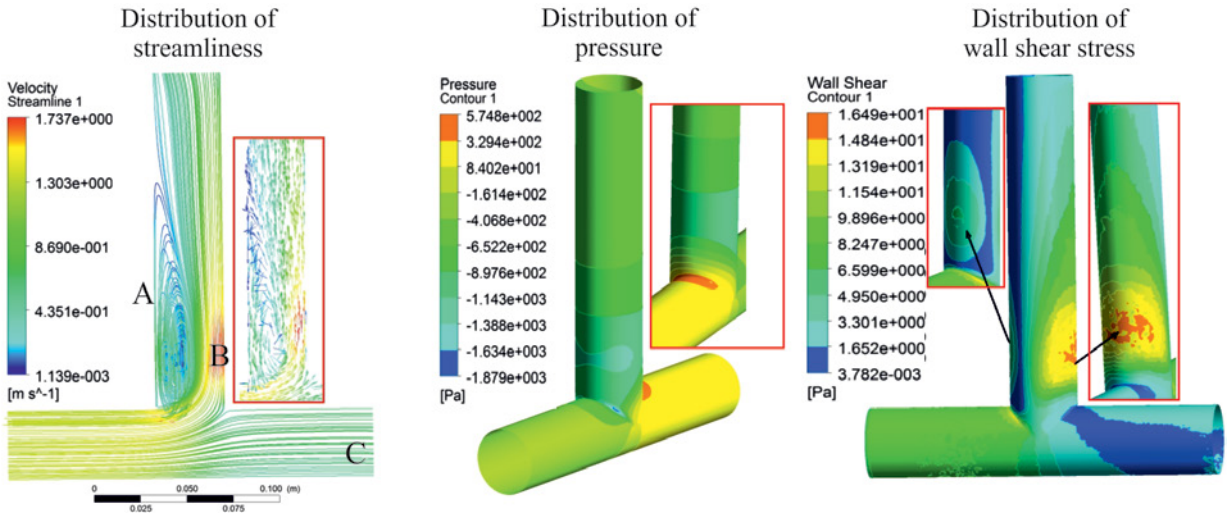


Fig. 6 - The results of numerical investigations on the tees.

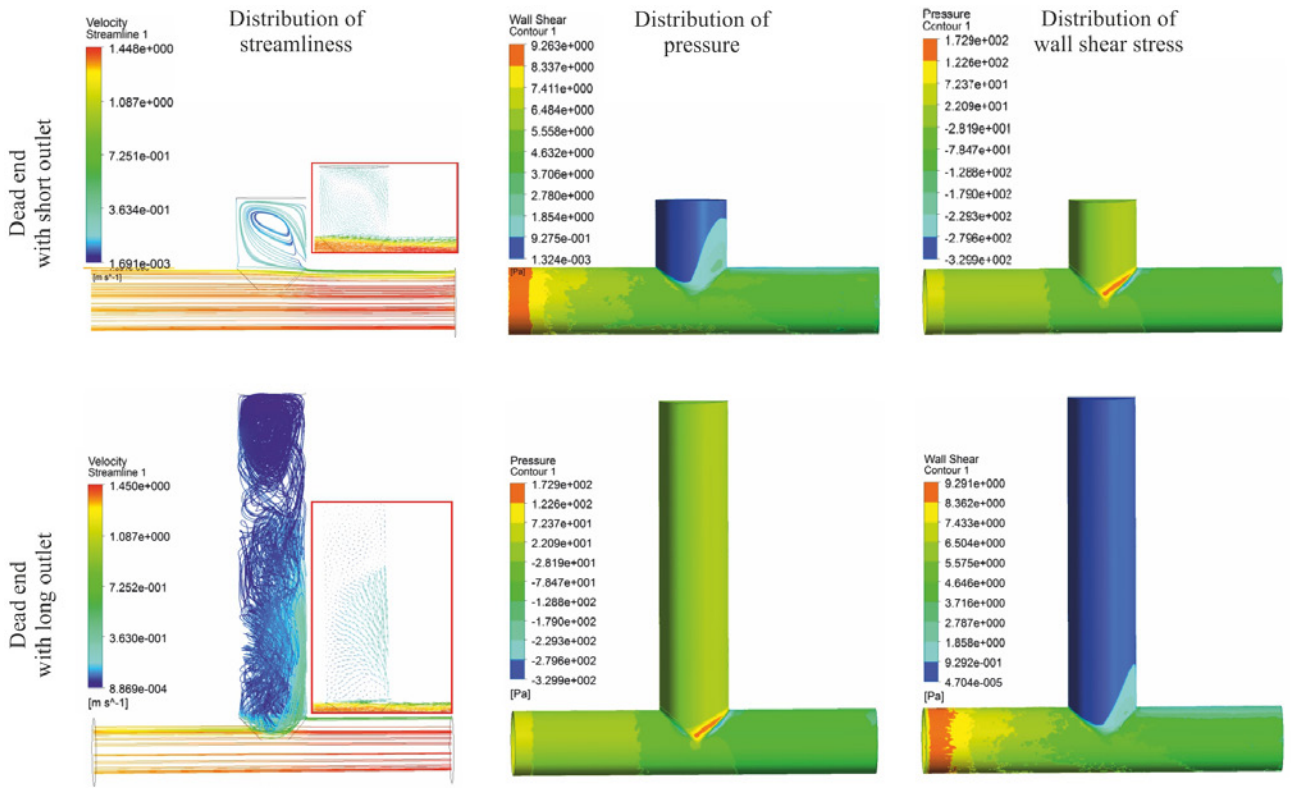


Fig. 7 - The results of numerical investigations on the dead ends.

accumulated there will not be cleaned out and this will be a perfect place for the development of microflora. The presence of the dead end also causes a 50% decrease in flow velocity, to a value of $u = 0.5 \div 0.7$ m×s^{-1} in the extension of the pipeline (right tube), on its upper edge. This place also cannot be cleaned properly.

The highest values of shear stress for tees were achieved in areas where there is the greatest flow velocity, namely on an outer wall located perpendicular to the flow direction of the liquid (Fig. 6). In the case of the dead ends, the shear stresses are the greatest at the inflow of the liquid (Fig. 7). On this basis, two sampling areas were indicated in a tee, described as the wall of the tee at the inflow and the upper surface of the pipeline behind the branching at the outlet. In the dead end, three sampling areas were described as the wall of the tee at the inflow (A), the wall tee at the outlet (B) and the upper surface of the pipeline for branching during discharge (C) (Fig. 8). Experimental research into the cleaning process in the laboratory CIP cleaning station was performed for dead ends and the results are shown in Figure 9.

There was a clear difference between the effec-

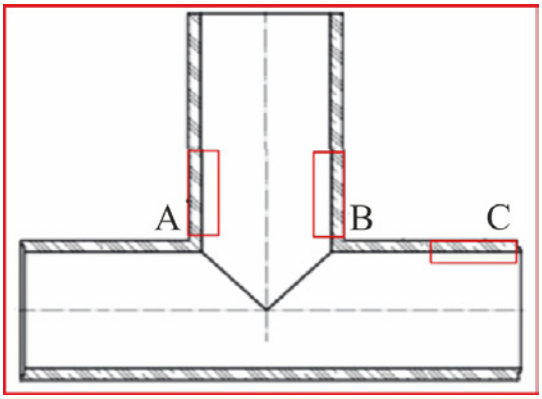


Fig. 8 - Some of sampling areas adopted for experimental research in blind ends: A - wall at the inflow; B -wall at the outlet; C - the upper surface of the pipeline at the outlet

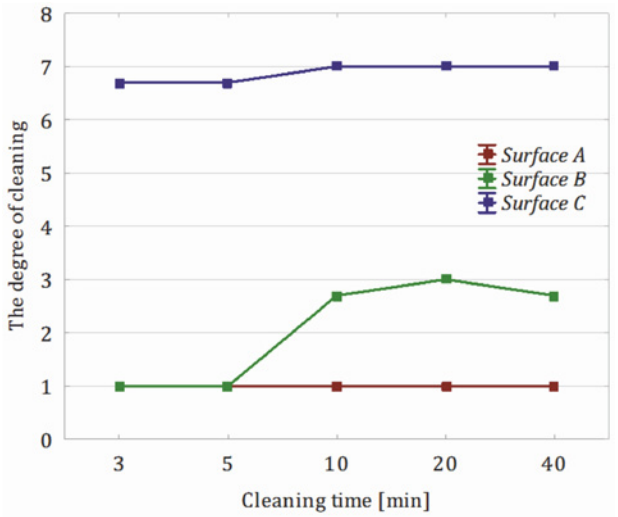


Fig. 9 - Each of the points represents the experimental research into the effectiveness of cleaning a dead end with a long outlet: A - wall at the inflow; B - wall at the outlet; C - the upper surface of the pipeline at the outlet.

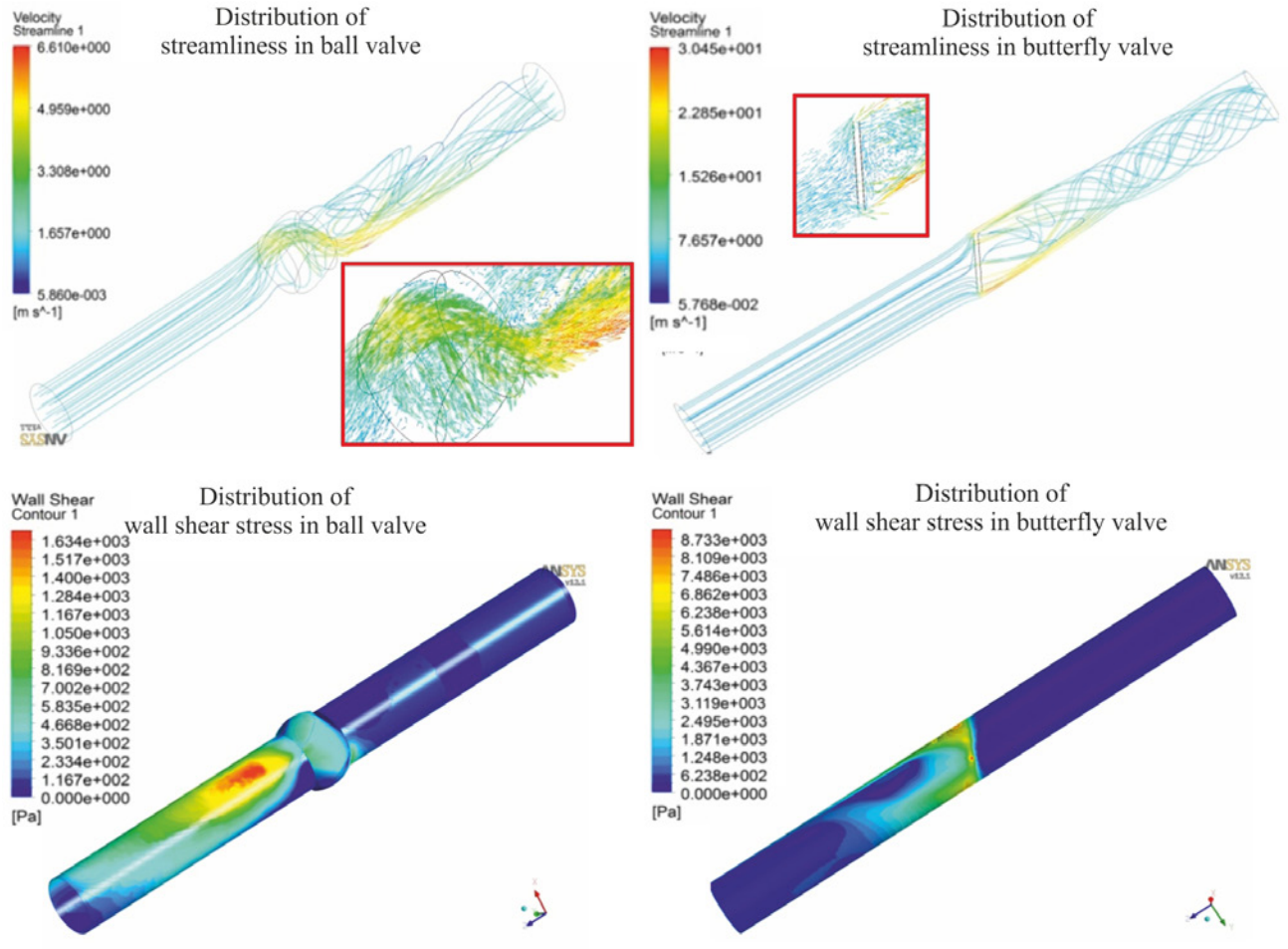


Fig. 10 - The results of numerical investigations on the valves.

tiveness of cleaning selected tee surfaces. A high degree of cleanliness was obtained in the upper surface of the pipe behind the branching from the outlet side. An additional increase in effectiveness was achieved by prolonging the cleaning process up to 10 minutes and this level was not substantially changed as a result of further prolonging the time to 40 minutes. This means that the lengthening of cleaning time improves its effect only to a certain level. Analysis of the results shows that those surfaces most difficult to clean are the ones on the walls of branching. The resulting degree of cleanliness for both surfaces did not exceed 4 cleanliness points. It was confirmed that the surfaces on which the fluid interacts with a lower velocity were cleaned with less effectiveness. In these places unclean sediment residue was also visible.

Butterfly and ball valves

The models of the fully open valve generate flow through a straight pipe (ball valve) and throw a pipe with an obstacle (butterfly valve). It was decided to simulate the unclosed situation of both valves. Numerical research into 30° open valves was carried out. For this position, the plate/bowl is turned 60° compared to the fully open state. The results indicate the formation of turbulence in the fluid in the area of the moving components of valves and flow discontinuities arise behind the valve (Fig. 10). The highest values of velocity and shear stress are achieved between the valve and the wall. Velocity values in these areas are as much as double the base values.

To assess the effectiveness of cleaning, the gasket surface and the ball surface (ball valve) and flaps (butterfly valve) were selected (Figs. 11 and 13). The obtained results of experimental tests are shown in Figs. 12 and 14.

In both of the assessed valves, sampling sites were the gasket and moving parts. In the case of the butterfly valve, the degree of flap cleanliness, with a prolonged cleaning process, increases from 3 to 8 points. In contrast, prolonged cleaning does not improve the degree of gasket cleanliness and from 10 minutes this remains at a similar level, around 4 cleanliness points. This confirms previous reports stating that the sealing elements are much more difficult to clean than parts made of stainless steel (EHEDG, 2003; LELIEVRE *et al.* 2003). This is not due to the cleaning solution flow conditions, but to the amplification of the

production pollution with gasket material. Only by strengthening the mechanical interactions of the fluid on the wall is their cleaning effect improved (BELMAR-BEINY *et al.*, 1993; JENSEN *et al.*, 2005; MIERZEJEWSKA *et al.*, 2013). In the case of the ball valve, the surface of the sphere in the entire research cycle was assessed as clean. Much worse is the situation on the join between the sphere and the surface of the pipeline. In the entire test cycle, this area was not properly cleaned and test results showed only 1 point of cleanliness. Differences in cleaning efficiency obtained in both valves result from their structure. In the case of the butterfly valve, poor cleaning results could have resulted from the presence of local flow resistance formed due to sectional shape changes and an obstacle unfavorable to the flow. Such an obstacle was undoubtedly the valve flap. The flow through the open ball valve is the flow through the pipe; hence, the well cleaned ball. However, the open ball valve completely covers the gaskets underneath and if soiling occurs, which does happen when the valve is closed, the cleaning solution does not reach the

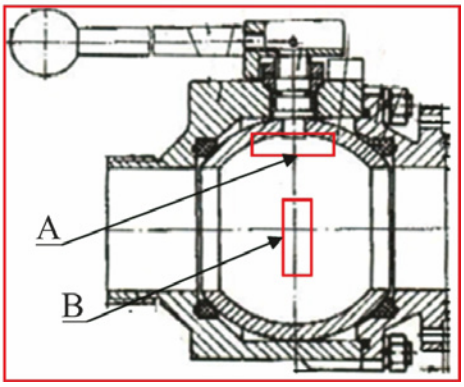


Fig. 11 - Some of the sampling areas adopted for experimental research into the ball valve: A - surface of the ball; B - gasket surface.

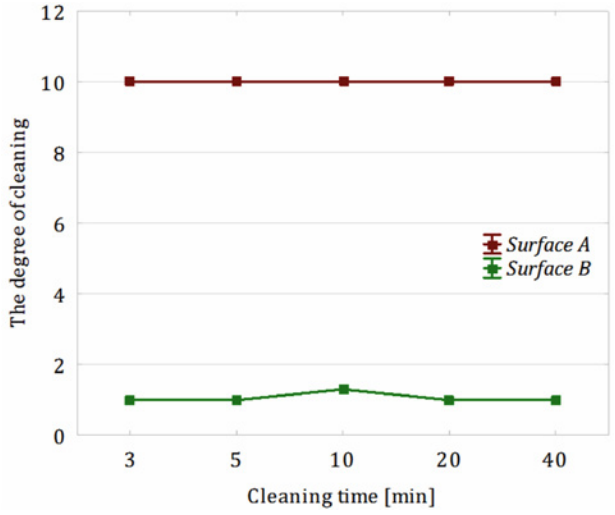


Fig. 12 - Each of the points represents the experimental research: A - surface of the ball; B - gasket surface.

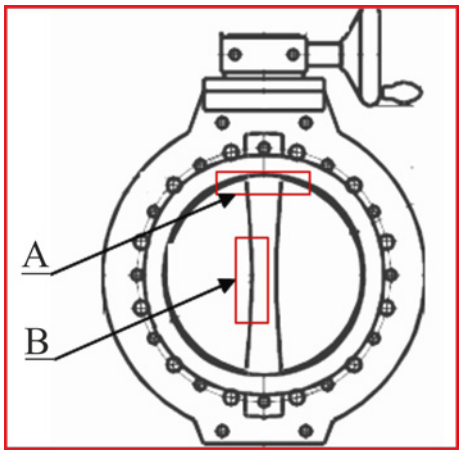


Fig. 13 - Some of the sampling areas adopted for experimental research into the butterfly valve: A - surface of the ball; B - gasket surface.

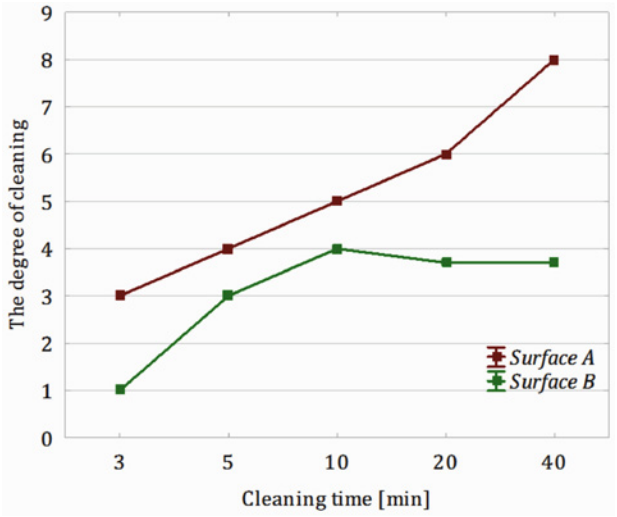


Fig. 14 - Each of the points represents the experimental research: A - surface of the flap; B - gasket surface.

seals and does not properly clean them, as was demonstrated in these studies.

CONCLUSIONS

The nature of the flow channel of a given device or installation has a significant impact on the hydrodynamic conditions of the fluid flow and, thus, the conditions of CIP cleaning. Local shear stresses, variable velocity profiles and pressure distributions explain the varying degrees of cleaning in standard CIP procedure on the surfaces of the researched pipe elements. This is consistent with studies by LELIEVRE *et al.* (2002b) and JENSEN *et al.* (2005). Identification of these areas was possible on the basis of the results of numerical analyses. The surfaces of elbows and tees were the most difficult to clean on the inner curves. Thus, their presence in pipeline installations should be as minimal as possible. In the case of valves, the moving parts and connection

with the gasket were hardest to clean. It was noted, however, that ball valves are easier to maintain clean and if there is the need, these valves should be used in transport systems. Risks associated with their improper cleaning can be further minimized by the use of appropriate construction materials. An innovation in this area is valves made of glass. Studies have shown that prolonging the time of influence of the fluid on the walls of the washed items does not improve the final cleanliness of the surface. Intensive removal of impurities occurs in the first minutes of cleaning and further increases in cleanliness are insignificant, as demonstrated in laboratory tests. From the point of view of process economy, a short cleaning time is much more beneficial, as it does not require the temperature of the cleaning liquid to be maintained for a long time and, thus, the production process is not idle.

The liquid flow in industrial transporting systems in closed arrangement prevents the location of unclean areas in the pipes. This was, however, possible by conducting simulated computer calculations using different computer methods.

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SURVEY

THE LOGISTICS OF DIRECT SALES: NEW APPROACHES OF THE EU

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ABSTRACT

The paper presents results of research on the direct sales or organized of food products through a visionary system of sustainable logistics as a strand of optimization processes with regard to transport, productive activity, and social well-being. We focus on some important aspects of direct sales, in particular, on environmental impact issues in the context of some of its various characterizations, and on some support instruments relevant to agricultural enterprises to foster to the latters some important conditions of competitive advantages with respect to the private aspects and the new recent public perspectives in the European arena.

- Keywords: direct sales, environmental impact, labelling, logistics -

INTRODUCTION

With respect to the topic of logistics in the agri-food sector, the interest of the national and international scientific community has focused in recent years on the *short supply chain,* with a transversal approach regarding transport, territory, productive activity (ZARBÀ, 2014), and social well-being (CHANG, 2012). This approach is then sustainable logistics, which takes into account not only economic aspects, but also the relevant social and financial security issues, as well as the environment (YAKOVLEVA, 2009).

Regarding the logistics of distribution systems' of *direct channels* with regard to direct sales (or organized direct sales), according of our knowledge, the contributions are limited.

The term *direct channels* refers to a set of configurations of production-consumption, often labeled with countless "names," and originates from farmer groups acting as local distributors to conduct direct sales (ALLEGRA, 2014).

The European Parliament[1] has launched a wide range of activities coordinated by the Commission in order to obtain a clearer picture of direct selling. These last activities concluded with the conference Local Agriculture and Short Food-Supply Chains, which provided the Commission with information essential to the preparation of the report to the European Parliament and the Council "on whether to establish a system of labelling on agriculture local and direct sales" (COM (2013) 866 final)[2].

In the Commission's report, the concept of *direct sales* is defined as meaning "sales by a farmer directly to a consumer, without intermediaries on the selling side"[3]. Therefore, the Commission, with respect to the direct sales, does not take into account the origin of agricultural products and foodstuffs traded, meaning the traded products do not necessarily have to have been cultivated by the same farmer, but may instead be obtained from other farmers. Therefore, this concept does not take into account the possible degree of integration of products from other origins[4], including from the local food system. Further, but not less important, in this sense, it does not specify the scope of the term *limited geographical area*.

The Commission seems, for the most part, to leave the evaluation, with respect to the origin of the product, to the same consumer, acknowledging the existence of a direct relationship between producers and consumers. In other words, trust and mutual understanding between farmers and consumers must exist for direct sales to take place.

In direct sales, the logistics sector assumes different configurations in each case, but nevertheless remains a cross-cutting feature and constant in these different configurations the physical handling of agricultural and food products from the firm to the point of sale. The farmers' markets involved in the direct sales generally have a limited production volume therefore the supply of the products is commonly carried out with *just-in-time* inventory management techniques.

From these considerations emerges the realization that, in those farms that practice direct selling, and regarding the placing of their products on the market, functions that govern the logistics (i.e., the sequence of operations in the phases of programing, production, and distribution) are based mainly on the organization and management of transport to the place of purchase.

The commercial area at local level, deputed to the producer-consumer direct sales, can be imagined as a sort of *hub and spoke*. These places, in fact, can be seen as the center of gravity of the sale of food products, which connects all the other satellite operations carried out by the interested parties. The food products will have to pass through the point of trade (or the *food hub*) to go from one party to another in the trade process. It is therefore a logistics system that relies mainly on transport by vehicles of varying sizes in relation to the functions performed.

An important issue that emerges from this logistics system, as shown in Fig. 1, is the reduction of the impact of food products transport,

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[1] European Parliament Resolution of September 7, 2010 on fair revenues for farmers: a better functioning food supply chain in Europe, P7-TA (2010) 0302.

[2] The Commission's report aims to "propose the adoption of instruments to support and promote farmer-managed food supply chains, short supply chains and farmers' markets, in order to establish a direct relationship with consumers and to enable farmers to obtain a fairer share of the value of the final sale price by reducing the number of middlemen and of the stages of the process."

[3] The Commission also dwells on the concepts *short food supply chains*, *local farming*, *local food systems*, and *local area*. In particular, *short food supply chains* means sales made by a farmer to a consumer through a small number of intermediaries, *local farming* means the production of agricultural products and foodstuffs with the aim of selling them in an area reasonably close to the farm of production, *local food systems* means that production, processing, trading, and consumption of food occur in a relatively small geographical area. Finally, the Commission designates the *local zone* a geographical area of such extension to be recognized by consumers as the origin of the product.

[4] In Italy, the matter is regulated by the Legislative Decree 228/200, article 4, paragraph 1, which reads as follows: "Farmers, individuals or groups, registered in the commercial register, as in the article 8 of the Law of 29 December 1993, n. 580, can sell directly to retail, throughout the territory of the Republic, the products that come mainly from their respective companies, observing the provisions in force regarding hygiene and health."

namely the reduction in the *consumption of mobility*, (i.e., CO_2 emission from production sites to those of the last kilometer of consumption and to those from the commercial space to the point of discharge of "waste"). Hence, actions aimed at achieving a better level of sustainable mobility are identified.

The pollution of the air due to transport of products through production chains has been the focus of numerous studies (Ministerial document, 2013)[5], which instead appear limited with respect to accidents, congestion (IANNONE, 2003), and noise (VAN PASSEL, 2010), perhaps in part because of the difficulties encountered in order to assess them (ISFORT, 2013). For the assessment of air pollution from the transport of food products for direct sales, the use of indicators represented in the label of the food miles (SILLING, 2009; VAN PASSEL, 2010; WYNEN, 2008) can be considered useful for analysis conducted on a short chain which identifies itself with the system of direct sales. It must also be remembered that the growth of direct sales initiatives, with the consequent competitiveness between producers, has gradually matured the need for tools aimed at obtaining favorable reviews by the consumer.

In essence, we are talking about a logistics that takes into account not only the physical movement of products, but also of the information flow in the reverse direction through the documental identification, in particular referring to fresh products of other origins and especially for the derivatives of vegetable and animal origin. The logistics is also capable of reducing the information asymmetry with ascending traceability techniques, namely, a labelling system in order to associate a symbol/logo with the qualitative characteristics of the food product to the producer. However, it is necessary to indicate that the Commission, in the above-mentioned opinion, believes that the establishment of a labelling scheme (possibly voluntary) in the direct sales, when direct communication is not possible, would "have limited impact".[6]

To study the logistics of direct sales of food products, the focus has been on organized structures of local farmers markets (MARINO, 2012)

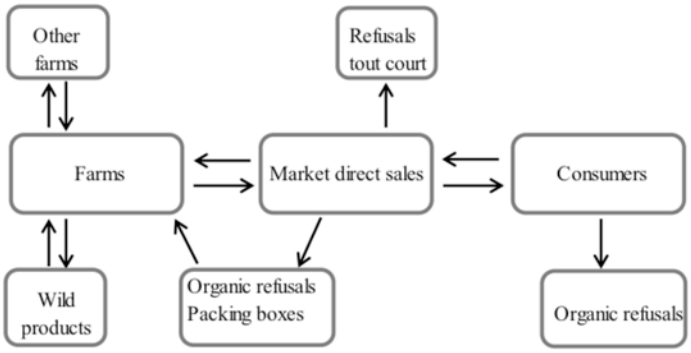


Fig. 1 - The logistics system of direct sales.

for "direct sales organized" between producers and consumers known at the national level. The related surveys have been conducted in Sicily, specifically in Catania and Ragusa, where agriculture (with its connected activities) and the corresponding local food distribution systems in the pertinent provinces assume different characters (D'AMICO, 2014).

Local agriculture accounts for 2.5% of Catania and 10.2% in Ragusa on the total value added of the Sicilian Region (75.9 billion euros) and these represent, among other things, the extreme values that include the other seven provinces. Even commercial distribution shows similar characteristics. In fact, the supermarkets are equal to 132 units in the province of Catania, and 40 in Ragusa, while hypermarkets are respectively equal to 10 units and one unit[7].

MATERIALS AND METHODS

The survey was conducted at of organized structures of direct sales in the cities of Catania and Ragusa of the Sicily. The sample consisted of 30 farmers, equally chosen in those cities, in relation to the regularity of their presence in the market and the agri-food products cultivated primarily by those farmers. With reference to the purchasers, the surveys included only customers who had bought food (250 people in Catania, 100 people in Ragusa).

[5] The analysis of the impact of transport mobility is based on the consumption of energy and/or carbon emissions with the application of indicators according to the methods of food miles and the Life Cycle Assessment or *LCA*, widely used in the scientific literature. The first approach is based on calculating the distance between the transport points, while the second approach is more holistic, complex and accurate. The method of LCA is used, in fact, to supplement and verify the impact of the simple measure of the mileage travelled as an indicator of the sustainability of logistics of chains, but at the same time extended to other impacts generated by the other stages of the production process (consumption of non-renewable resources, the use of means of production impacting soil, water and air, etc.) from the producer to the point of sale of the products, even from influential external factors/elements (BALDO, 2008).

[6] The Commission points out also that the Eurobarometer[6] survey on the exercise of the rights of the consumer, has revealed the lack of knowledge and skills on the part of consumers, including with regard to the interpretation of labels and logos.

[7] Assessorato Regionale dell'Economia – Servizio Statistica ed Analisi Economica. 2012. "Relazione sulla situazione economica della Regione Siciliana", Annuario Statistico Regionale.

The markets take place weekly, on Sundays in Catania on Saturdays in Ragusa (i.e., when there is a high turnout of consumers at supermarkets of the modern agricultural and food large retailers).

The interviews were conducted in 2013 with the help of a closed-ended questionnaire divided into two sections, the first addressed to the producers, the second to the consumers. Among the questions formulated to obtain knowledge about the logistics of "direct sales organized," in particular, those related to vehicles used for transport, to and from the farmers market and especially the role of food products with hard to trace origins.

For the analysis of the transport logistics of food products in the farmers markets, in relation to the generation of the negative externalities, we took into account aspects such as the opportunity to not make the distinction by species and variety of fresh products, in relation to their wide variability, both quantitative and qualitative, partly due to the seasonality that characterizes them; this also applies to the distinction between products made with environmentally friendly production and conventional methods[8].

The reasons originate from the difficulty in determining the replenishment coefficient (t-km) for each product comprising a delivery load (commonly transported as mixed loads), and for both outbound and return trips. In the same way, it is rather complicated to define the logistics of the return of unsold products, packaging for recycling, and waste. The question also applies to the journey to and from the places of supply of other food products from competing firms, or of edible wild vegetables harvested by the same farmers (ZARBÀ, 2013).

In addition, the difficulty of quantifying the composition of expenditure by the buyer for each product (numerical coefficient) should be considered.

And no less important is the transport for disposal of waste (general waste, scraps, non-recyclable packaging, etc.), when this service is public, from the farmers markets to the public landfill. The complexity of the above factors revealed, about the procedures for determining air pollution, induce us to evaluate the logistics indicators related to the transport not for individual food product but for individual driver of the vehicle[9].

Accordingly, the main element that influences the efficiency of transport logistics from the environmental point of view is the evaluation of modal transport in the action range of the farmers markets, that is customarily spread over short distances, in particular, with light vehicles, both commercial or not. It has been necessary, therefore, to identify those factors and/or elements that cause CO_2 emissions (only) by the burning of traditional fuels[10].

For the determination of CO_2 emitted per km/passenger, useful information has been taken from the *Guide 2013 to Save Fuel to the CO_2 Emissions From Cars*[11], from EU regulations about the compulsory reduction of CO_2 emissions of vehicles[12] and from databases[13] that provide average values of emissions (g/kg) for the different classes of vehicles.

For the definition of the average amount of CO_2 produced and emitted into the air due to transport (and so to the logistics of direct sales), we proceeded as follows:

kg CO_2 vehicle = km performed x 2 x C
(where C is the emission factor)

These factors relate to the use of the suburban mean; in the case of the urban route, it would be necessary to increase the factors in proportion to the increase in the consumption (an increase of approximately 30%) (BALDO, 2008).

Another issue addressed was the logistics of direct sales in terms of traceability of products, the question raised by the Commission regarding the validity or not of the use of labelling with a logo/symbol for universal recognition of the EU's system of direct sales, also considering the related costs that would result if it were to be established.

In addition, by correlating other collected data, other qualitative elements of judgments on the existence of and prospects for growth and development of the distribution systems for direct sales have been developed.

Thus, alongside the indicators so far observed, additional factors related to logistics can act on the sustainable aspects of direct sales, albeit with indirect actions. In order to assign a val-

[8] This is to avoid, among other things, the different influences of the impact on the environment and therefore, on sustainable logistics upstream in the supply chain.

[9] The low value of the mean of the producer and buyer coefficients determines a high value of emissions.

[10] It is necessary consider that, limited to logistical steps from transport in urban areas, the impact of air pollution should also be evaluated in terms of emissions of particulate matter (PM10) and nitrogen oxide (NOx) by type of variables fuel.

[11] Ministerial document of the Ministry of Economic Development in cooperation with Ministry for the Environment, Land and Sea and the Ministry of Infrastructure and Transport"

[12] Performance standards for emissions from new passenger cars as part of the community's integrated approach to reduce CO_2 emissions from light-duty vehicles.

[13] Among those consulted we mention, Quattroruote ECO, "Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA) e "CO2nnect."

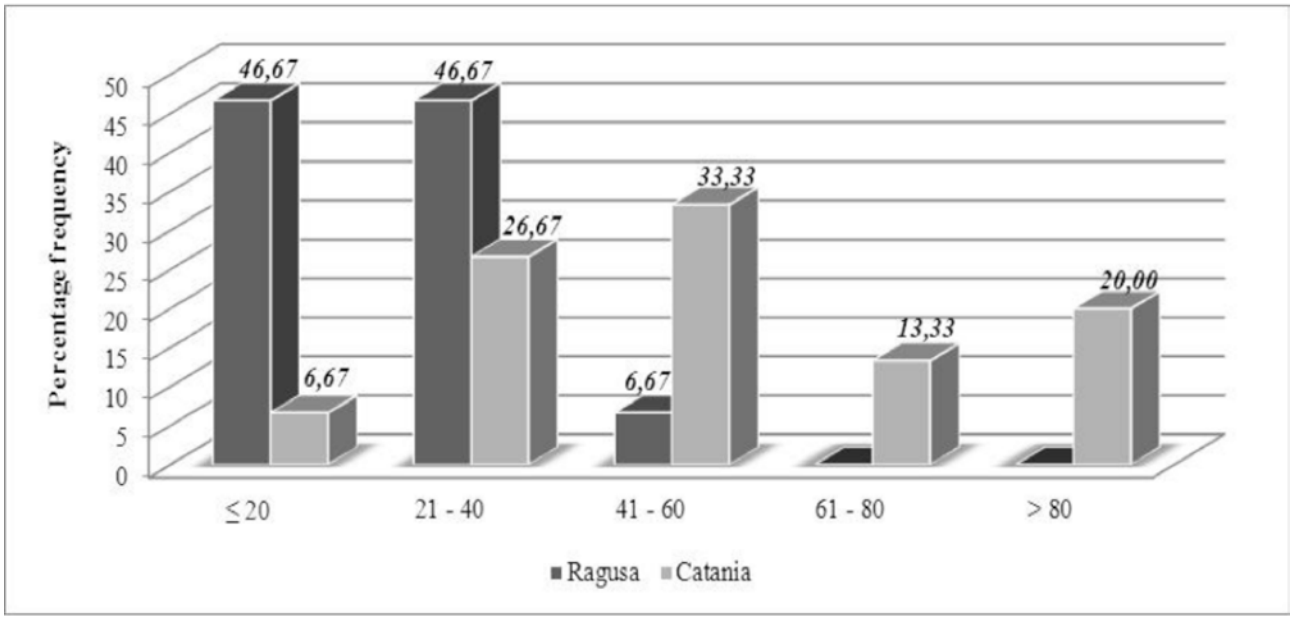


Fig. 2 - Frequency percentages relating to distance from farms for farmers market for the classes kilometers.

ue common to these indicators, we considered the units observed in a single view, in order to achieve more meaningful results on the socio-economic aspects of direct selling outright. In particular, a total sample of 30 farmers on the one hand, and on the other hand a sample of 350 randomly selected customers outside the market, were included in the study.

The measured indicators were elaborated by computer in order to provide synthetic coefficients to determine the best strategy for the issues highlighted.

RESULTS AND CONCLUSIONS

The distance separating the farmers from the agricultural companies and the farmers markets frequented, is quite variable, as can be noted from Fig. 2. However, the *paths connected*, is extremely variable owing to a series of common supply factors (plots outside the company, the purchases of products by competitors, the gathering of plants wild, edible, etc.) for collateral services (re-fueling, etc.), and for the rural and urban nature of the path. However, with re-

Table 1 - Distribution of consumers for the path mode for the farmrs market for the classes kilometers (*)

Classes kilometers	≤ 1,00		1,01 - 5,00		5,01 - 10,00		> 10,00		Total	
Path mode	Ragusa	Catania	Ragusa	Catania	Ragusa	Catania	Ragusa	Catania	Ragusa	Catania
				Number of consumers						
Walk	34	67	2	25					36	92
Bicycle	4		2	1	2				8	1
Moped	2	4	1			2			3	6
Motorcycle		12	1	15		12		1	1	40
Car	20	42	22	34	2	17	4	14	48	107
Bus	1		2			4	1		4	4
Total	61	125	30	75	4	35	5	15	100	250
				Percentages						
Walk	55,7	53,6	6,7	33,3					36,0	57,6
Bicycle	6,6		6,7	1,3	50,0				8,0	3,2
Moped	3,3	3,2	3,3			5,7			3,0	7,2
Motorcycle		9,6	3,3	20,0		34,3		6,7	1,0	
Car	32,8	33,6	73,3	45,3	50,0	48,6	13,3	93,3	48,0	26,4
Bus	1,6		6,7			11,4	3,3		4,0	5,6
Total	100,0	100,0	100,0	100,0	100,0	100,0	16,7	100,0	100,0	100,0

(*) Our elaborations on directly acquired data

gard to consumers, most of them reside within a short distance from the market, enabling them to reach it by foot or bicycle, as can be noted from Table 1.

Applying the emission factor to the kilometric distances traveled by the interviewees, expressed in g/km CO_2, it was possible to estimate the relative air pollution, with reference to producers (Fig. 3) and consumers (Table 2).

The corresponding values, although approximate, highlight the importance of the phenomenon, as they can be considered useful to determine the eventual overall emission of CO_2 when the market is active.

Turning to the socio-economic indicators, the factor *liquidity* is the main stimulus that encourages farmers to "direct sales" (Table 3), especially with regard to plant products; the seasonality marks out that these products does not always allow farmers to have money flows applicant so to reduce the amount of the capital of anticipation.

Some factors contribute to the levels of profitability (*searching of profit* and *certainty of sale*) and increase in the value-added services (*logistics support services to the customer* and *sales method*). However, others act on the psychological segmentation (*autonomy*, *type of customers*, and *image*).

For consumers (Fig. 4), the indicators *seasonality*, and *quality of products*[14] prevail, as was expected, over all other *stimulating factors*, i.e., on *experiential variables*, on *relationship with the farmer*, and finally over *proximity to domicile*; in fact, the distance from the home of the consumer to the markets is not an obstacle, especially if parking is readily available. Adequate parking is considered by consumers to be one of the most important factors (Table 3) inducing them to purchase at supermarkets, if for no other reason that fringe of consumers[15] who said they continue shopping at the large retailers on the same day after being to the direct sale market.

Statistical parameters	Catania	Ragusa
N	15	15
Mean	56,53	24,29
Standard deviation	25,21	11,83
Minimum	19,50	1,00
First quartile	31,70	18,40
Median	60,80	22,57
Third quartile	73,20	32,70
Maximum	100,00	51,70

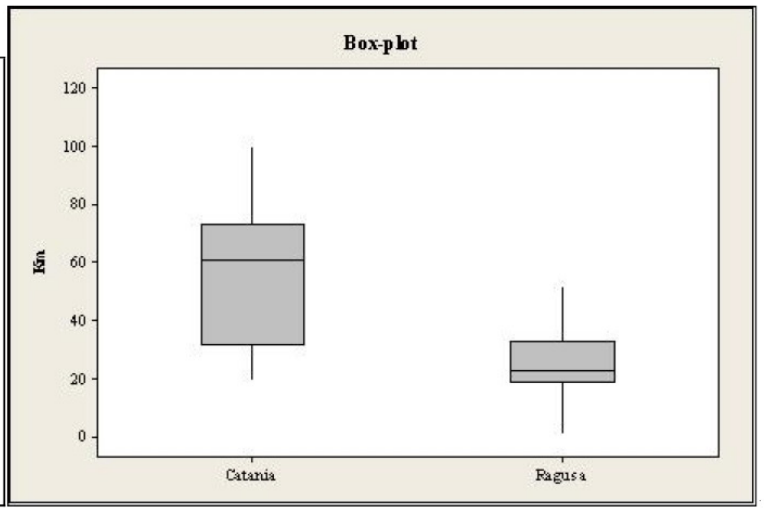


Fig. 3 Distance from the farms to the farmer's market. The emission factor equal to light commercial vehicles (vans) = kg/km 0.175.

Table 2 - Distribution of the CO2 emitted for classes kilometers and vehicle type used of the consumer (*)

Classes kilometers	≤ 1,00		1,01 - 5,00		5,01 - 10,00		> 10,00	
	Ragusa	Catania	Ragusa	Catania	Ragusa	Catania	Ragusa	Catania
Vehicle type	Kg CO2	Kg CO2	Kg CO2	Kg CO2	Kg CO2	Kg CO2	Kg CO2	Kg CO2
Moped	0,136	0,544	0,340		2,040			
Motorcycle		1,128	0,470	7,050		16,920		5,640
Car	2,660	5,586	14,630	22,610	3,990	33,915	31,920	111,720
Bus	0,069		0,690			4,140	4,140	

(*) Our elaborations on directly acquired data
The emission factor equal to: moped = kg/km 0,068; moyotcycle = kg/km 0,094; car = kg/km 0,133; bus = kg/km 0,069

[14] For these factors the maintenance of distinctive organoleptic characteristics of food products, the respect for time to market, or "times logistic" (LANINI, 2006) becomes of major importance.

[15] In particular, belonging to the age group 35-55 years.

This *bipolarity* for purchases favors the increase in the proportion of waste of agro-food products that is generated from supermarkets.

The question of waste and food wastefulness generated by the trade in direct channels is not a significant factor of consideration[16], because the volume of waste is fairly low.

With regard to the labelling of food products, 76.7% of farmers recognizes the utility of labelling, while the remaining 23.3% believes that it is not necessary. With reference to the options shown in Table 4, farmers aim more on the "logo", as long as private and less on the use of a "descriptive table"[17]. Consumers express themselves in the same direction as the farmers consumers and agree with farmers. In fact, the con-

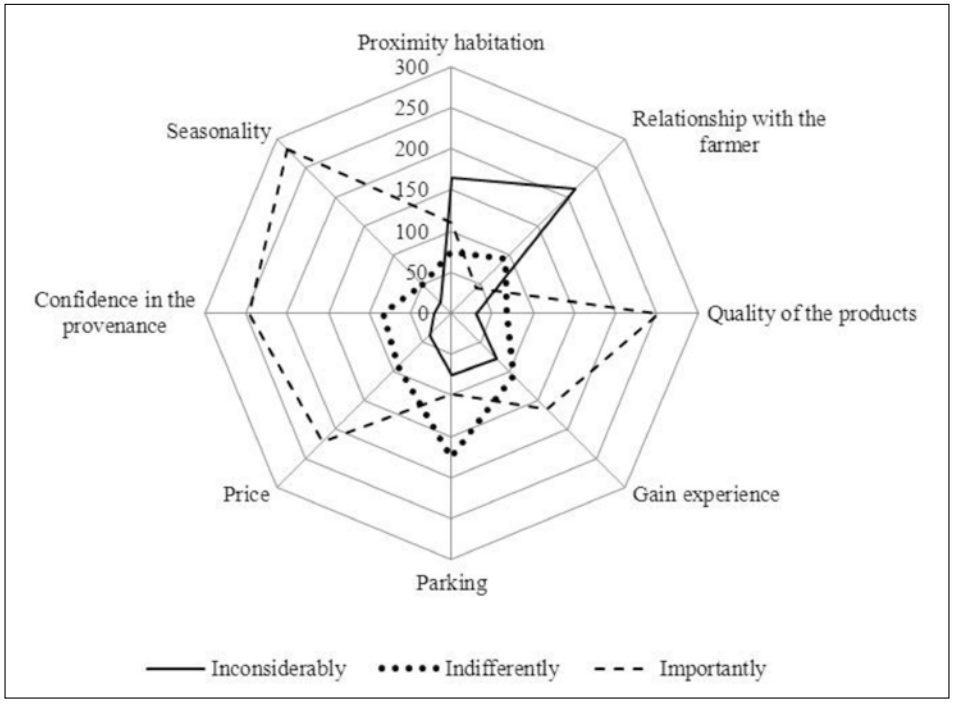


Fig. 4 - Factors affinity for farmers' market by consumers.

Table 3 - Distribution of the producers of food products to the major factors of stimuli for direct sales (*)

Stimulating factors	Completely concordant		Fairly concordant		Shortly concordant		Not at all concordant		I do not know	
	N.	%	N.	%	N.	%	N.	%	N.	%
Search for profit	16	*53,3*	9	*30,0*	3	*10,0*	1	*3,3*	1	*3,3*
Search for profit	16	*53,3*	11	*36,7*	1	*3,3*	1	*3,3*	1	*3,3*
Liquidity	25	*83,3*	2	*6,7*	3	*10,0*				-
Autonomy	17	*56,7*	1	*3,3*	9	*30,0*	2	*6,7*	1	*3,3*
Direct relations	10	*33,3*	12	*40,0*	8	*26,7*				-
Image	10	*33,3*	8	*26,7*	9	*30,0*	1	*3,3*	2	*6,7*
Method of sale	8	*26,7*	7	*23,3*	7	*23,3*	2	*6,7*	6	*20,0*
Kind of customers	3	*10,0*	2	*6,7*	7	*23,3*	10	*33,3*	8	*26,7*

(*) Our elaborations on directly acquired data

[16] It must furthermore be considered that under the regulation of the market organized, the farmer's task is to dispose of the residues of the sale (organic remains are subjected to composting or used as fertilizer or animal feed).

[17] This indeed is not much in accordance with what is expressed by the Commission (COM (2013) 866 final) in its report to Parliament about the "optional quality terms" that manifesting through a text, "is an effective tool with which farmers can communicate the added value conferred by their products and make sure that these extra efforts could be rewarded".

Table 4 - Distribution of producers and consumers for the opinions expressed by types of labelling (*)

Specification	Completely concordant				Fairly concordant				I don't know			
	Producers		Consumers		Producers		Consumers		Producers		Consumers	
	N.	%	N.	%	N.	%	N.	%	N.	%	N.	%
Labelling	14	*46,7*	131	*37,4*	9	*30,0*	158	*45,1*	7	*23,3*	61	*17,4*
- Logo	10	*71,4*	70	*53,4*	6	*66,7*	100	*63,3*				
brand factory	*5*	*50,0*	*37*	*52,9*	*2*	*33,3*	*100*	*100,0*				
region of Sicily	*3*	*30,0*	*18*	*25,7*	*2*	*33,3*						
EU	*2*	*20,0*	*15*	*21,4*	*2*	*33,3*						
-Descriptive table	4	*28,6*	61	*46,6*	3	*21,4*	58	*36,7*				

(*) Our elaborations on directly acquired data

sumers did not favor the use of a mark at the regional level and of any other co-existing logo/ symbol at the community level[18].

The local bargaining is characterized by the direct relationship between producers and consumers, and it is this relationship that would negate the need for the labelling of food products (especially if fresh and seasonal), and consequently would not induce to the related logistics activities. Therefore, we wonder how the consumer can recognize, in the absence of a "label"; the origin of the goods offered for sale by the farmer, which originate from other sources.

The results have allowed us to specify the characteristics of logistics that involve not only the handling of materials and its environmental impact of food transport, but also other important functions, which are recognized as playing an important role for an efficient distribution system based on direct sales.

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[18] Accordingly, it concerns the use of "a possible instrument at EU level" (COM 2013, 866 final).

DOES SMART AGRICULTURE GO DOWNSTREAM IN THE SUPPLY CHAIN?

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ABSTRACT

Nowadays it seems to be imperative for smart farms to go downstream in the Supply Chain (SC) to supplement the revenues of their underlying product sales. Using the well-structured database of Eurostat Supply tables for EU-27, this study intends to verify what products the Agricultural sector is offering beyond its core business activity to diversify its portfolio. The findings demonstrate that Agriculture is going downstream in the SC, predominantly moving towards its traditional activities: food processing, agro-tourism, and recreation. Green energy is the newcomer. Smart farmers should innovate in the mainstream of their secondary activities, using the leverage of lucrative activities and rethinking their vertical integration.

- Keywords: agricultural portfolio diversification, farmer choices, supply chain, growth and share matrix, supply tables, secondary production -

INTRODUCTION

Research on Food Supply Chain (FSC) entails various difficulties. Prevailing among these is the quantification of the production value obtained by agriculture from outside its core activities, such as from: food processing, accommodation and food services, wholesale and retail, and other services. These concerns are general problems that likewise involve every other industry. When evaluations are done, they often appear to be very partial (being limited to a specific product e.g. an organic one), unstructured (e.g. applying un-standardised and non-homologated classifications), and suffering from duplication (e.g. the turnover from direct sales which is already included in the value of primary agricultural materials, as noted in KEAFSEY *et al.* (2013). What is needed, therefore, is the provision of a complete, standardised and certified database, without any duplication, specifically such as the one described hereafter. Over the last 5 years, Eurostat has disseminated a new set of input-output tables, named Supply and Use, in conformity with NACE and CPA classifications within the framework of the ESA-95 system, and harmonised with the UN-System of National Accounts and the ISIC classification (EUROSTAT, 2008). In this database, the Supply table provides the value of core business production for each industry, as well as of secondary productions that are, in the main, core competences of other sectors. Contextually, the Supply table provides the value of a group of homogeneous products (e.g. Agricultural products) supplied to the economy as a whole by each sector, broken down by the industries that deliver it as a principal product. Two annual time series have been utilised: the first encompasses the 2000-2007 period, and was compiled using the NACE rev. 1.1 classification, while the second has recently been published (in 2013) according to the NACE rev. 2 classifications, and contains EU-27 and EA-17 tables for 2008-2009, as well as with data limited to 12 EU countries for 2010. This study utilises the first series for an inter-temporal comparison (2000-2007), and the second series for an up-to-date evaluation relating to the last available year (2009).

The study objectives are as follows: 1) to quantify the value of activities included in the Agriculture portfolio that are classified by Eurostat as primary and secondary production, which pertain to the core business of other industries; 2) to create a Boston Consulting Group (BCG) Growth-Share Matrix in order to verify farmers' choices and expectations regarding downstream paths of diversification in the FSC (HENDERSON, 1970); and 3) to evaluate the difficulties facing Agriculture in its attempt to diversify its portfolio, and offer suggestions about potential opportunities in this regard.

MATERIALS AND METHODS

This study utilises the EU-27 Supply matrixes that reflect the primary characterising and secondary non-characterising production activities of industries. Generally, survey results mainly concern enterprises with numerous secondary activities, and it is the principal activity of an enterprise that determines its allocation to a specific industry classification. The columns of the Supply matrix present the production program for each industry, including the output of its primary and secondary productions. For each bundle of products listed in the rows (following CPA classifications), it is possible to find the industries that produce those goods as their primary or secondary production listed in the columns. The principal activity or production of an industry is reported on the diagonal of the Supply matrix while secondary activities are listed off the diagonal (EUROSTAT 2008).

For the period 2000-2007, the European tables, published up to 2012, were using the NACE rev 1.1 Classification of Economic Activities. Until 2004 the geographical reference was EU-15 countries, and EU-25 or EU-27 thereafter. The national accounts domain as a whole has implemented the NACE rev 2. Classification of Economic Activities (harmonised with UN ISIC rev. 4), and has applied this since the 2008 reference year. The latest input-output tables were published in 2013, and concern the 2008 and 2009 years for EU-27 and EA-17 countries. These tables have been built on the basis of the new industry classification.

The first part of this study refers to the activities (included in the NACE rev.2 classification under Section A, Division 01) denoted as "Crop and animal production, hunting, and related service activities", which encompass seven groups, articulated as follows: 01.1 Growing of non-perennial crops; 01.2 Growing of perennial crops; 01.3 Plant propagation; 01.4 Animal production; 01.5 Mixed farming; 01.6 Support activities to agriculture and post-harvest crop activities; and 01.7 Hunting, trapping, and related service activities. These groups are then further articulated in 25 classes (EUROSTAT, 2008a). Secondary activities/productions are all those which are not included in the above definitions, and which are appropriately classified under other industries/groups of products, according to the same NACE rev. 2/CPA classification.

After having calculated the economic importance of primary and secondary Agriculture production for EU-27 (2009), articulated by the core competences of industrial sectors, the methodology of BCG has been applied. This last consists of the construction of a Growth-Share Matrix (GSM), modified appropriately for the purposes of this study. For EU-27 in the 2000-2007 period (adopting the old NACE rev. 1.1 classification), the compound growth rate of each ex-

tra-agricultural production (y-axis of the GSM graph), as well as the logarithm of its share (in %) of agricultural secondary production (x-axis of the GSM graph), has been calculated for 2007. This is an alternative application of GSM, since the aim is to establish the position of agricultural secondary activities in the farmers' portfolio rather than their competitive position in the destination market. However, the results obtained have allowed the classification of Agricultural secondary production under the 4 classical typologies from left to right on the graph, being: a) 'Stars', which are fast-growing investments by Agriculture outside its core business which have a high impact on its secondary activities. A star might only be cash-neutral, despite its strong position. Large amounts of investment may be required to defend their position against competitors; b) 'Question Marks' are fast-growing investments with low share of Agricultural secondary production. Substantial net cash input is required to maintain or increase their production share; c) 'Cash cows', that have a high secondary production share, but are slow growing. These should generate substantial cash inflows ready for use in other investments; and d) 'Dogs', that are secondary productions with low production share and slow-growing investments which generally regard mature product in the final phase of life-cycle. These investments tend to have a negative cash flow, which is likely to continue. However, these typologies are only the result of past investment trends by Agriculture in specific extra-agricultural activities. They do not provide indications of a farms' capability to successfully develop new strategic paths for further downstream diversification in its portfolio of secondary products in the FSC. To overcome difficulties, there is a need for the height of the entry barriers in the destination markets to be determined through specific indexes that are described below. In the literature there are other barrier indexes, but these are not suitable for the purposes of this study (ORR, 1974; MANN, 1966). The originality of the Chang indices (compare CHANG and ISEPPI, 2012) is essentially that: (i) each industry/country is compared using a reference system of economic or geographical average behaviour; (ii) the symmetry is fundamental: it considers both the insulation ability of each sector in building up entry barriers, and the invasion ability of other sectors to enter the core business area of the reference industry; and (iii) it also highlights the balance between the performance of entry barriers and the invasion ability. These indices have been applied in order to determine whether the markets of industries into which Agriculture is entering and hoping to expand market share have high or low entry barriers that prevent or allow penetration. These indices allow for both an ordering among individual sectors and individual countries, and for the investigated phenomena to be measured. For each country (and for the complex of countries), the Supply matrix is taken by product and by industry. For a given industry i (n is their number), P_i denotes the proper production (namely the production in the industries' primary field of competence), S_i represents the industries' secondary production in all the remaining group of products, and A_i is the total secondary production of all the other industries involved in the core business of the given industry i. It is necessary to normalise the indexes, specifically:

p_i is P_i normalised by $\sum_{j=1}^{n} Aj$ (1)

$a_i = A_i$ normalised by $\sum_{j=1}^{n} Pj$ (2)

$s_i = S_i$ normalised by $\sum_{j=1}^{n} Sj = \sum_{j=1}^{n} Aj$ (3)

The first index is the Chang Entry Barrier Index:

$$\Phi_i = \frac{\lg \frac{a_i}{p_i}}{1 + \left| \lg \frac{a_i}{p_i} \right|} \quad (4)$$

The value ranges from -1 to +1. If $a_i = 0$, no penetration happens, hence the index attains the maximum +1. The minimum -1 represents a theoretical case limit in which the entire production of an industrial sector is the secondary of the other sectors.

The second index is the Chang Invasion Index.

$$\sum_{j=1}^{n} Pj$$

This compares the differences between external secondary activities and internal (incoming) secondary activities, and normalises them with the whole of the balance of secondary activities for all the sectors.

The Chang Invasion Index is:

$$I_i = \frac{(n+1)(S_i - A_i)}{\left(\sum_{i=1}^{n} |Sj - Aj| \right) + n|S_i + A_i|} \quad (5)$$

(refer also to index 13 in CHANG and ISEPPI, 2011). The index ranges from -1 to 1.

"Of course negative values mean that the invasion undergone by the industry is greater than the penetration it performs, 0 (zero) means balance, positive values mean that it expands more than it is invaded. Thus the index is not only connected to entry barriers, but also tied to the capability or interest to overcome the entry barriers of other sectors" (CHANG and ISEPPI, 2012).

RESULTS

In the EU-27 whole economy (2009), total domestic production at basic prices amounts to €22,028.66 billion. Adding imports of €1,465.66 billion and Direct purchases made abroad by residents, and Subtracting the Cif/fob adjustments on imports, it is possible to obtain a figure for EU total supply of goods and services of €23,649.94 billion, including exports. In respect to domestic production, the percentage share of primary production, corresponding to the sum of core business activity of every industry, accounts for 92.6% of the total production, with a total value of €20,398.84 billion. EU-27 Secondary production amounts to only €1,629.82 billion, corresponding to 7.4%.

In this context, the industry named by NACE rev. 2 as "Crop and animal production, hunting, and related service activities" (hereafter Agriculture) has a total production of €350.42 billion, of which €327.16 billion pertains to primary production (93.36%), and €23.25 billion (6.64%) to secondary production. As can be seen, the incidence of primary production of the Agricultural Industry is above the EU average (93.36% *vs.* 92.6% respectively), whilst the contrary is the case for secondary production (6.64% *vs.* 7.4% respectively). This implies that Agriculture is less able and capable to undertake activities outside of its core business than the mean for the economy as a whole. At the same time, the group of products (CPA), named Products of agriculture, hunting, and related services (hereafter Agricultural Production), derives its total production from the activities of both Agriculture and all other industries. The value at basic prices of agricultural products produced by other industries amounts to only €3.36 billion, being only 1.02%, of total Agricultural production, whilst Agriculture produces the overriding part (98.98% *vs.* 92.60% of the whole economy production). This is a clear signal that agricultural activity is very difficult to undertake, and that exogenous and endogenous barriers to entry in the agricultural core business are very high. In synthesis, EU Agriculture has the potential to enter the field of primary activity of other industries, but until now it has not even been able to achieve the average share of diversification of the economy as a whole. In the meantime, the above result demonstrates that entering farming's core business is very hard for most firms.

Regarding the diversification of the Agricultural industry, ten groups of products absorb the major part of its efforts, considering those that furnish at least 1% of its secondary production (Table 1). These product groups represent 94.8% of Agriculture secondary production, whilst the remaining 55 groups of products together represent only 5.2%.

The principal fields of secondary activity in EU-27 Agriculture are Food, beverages, and tobacco products, whose value in this field are worth €15.83 billion, being about 68.1% of farmers' total Secondary production. This is a signal that European farms are pursuing an obsolete model of downstream vertical integration, already dismissed by the same manufacturers who now identify the most lucrative downstream activities specifically as services provision rather than transformation of raw materials such as agriculture. This is the profit imperative! (WISE and BAUMGARTNER, 1999).

Table 1 - Agricultural Industry Secondary and Primary Production in the Supply Chain (EU-27, 2009).

No	Code	Secondary Products (CPA) of Agricultural Industry	Million of Euro	% Share
5	CPA_C10-C12	Food products, beverages and tobacco products	15,830	68.08
36	CPA_I	Accommodation and food services	1,265	5.44
27	CPA_F	Constructions and construction works	1,178	5.07
51	CPA_N77	Rental and leasing services	981	4.22
29	CPA_G46	Wholesale trade services, except of motor vehicles and motorcycles	967	4.16
31	CPA_H49	Land transport services and transport services via pipelines	461	1.98
60	CPA_R93	Sporting services and amusement and recreation services	424	1.82
54	CPA_N80-N82	Security and investigation services; services to buildings and landscape; office administrative, office support and other business support services	367	1.58
24	CPA_D35	Electricity, gas, steam and air-conditioning	339	1.46
40	CPA_J62_J63	Computer programming, consultancy and related services; information services	226	0.97
		Agricultural Industry main fields of activities	**22,038**	94.78
		Other industries < 1% on the Total	1,214	5.22
		Total Secondary Production	**23,252**	100.0
		Principal Production	**327,163**	
		Total production of Agricultural Industry	350,415	

Moreover, Agriculture performs downward vertical integration with some service activities, but only marginally, and only in the fields in which it has some sort of traditional experience either in a conservative frame or in up-to-date evolution. These activities are: Accommodation and food services; Rental and leasing services; Wholesale trade services; Land transport services and transport services via pipelines; Sporting services and amusement and recreation services; Security and investigation services; Services to buildings and landscape; Office administrative, office support and other business support services; and Computer programming, consultancy and related services and information services. Construction and construction works are also in the experiential tradition of agriculture, while the activity of Electricity, gas, steam, and air-conditioning is a newer field of activity, encouraged by strong incentives for Green Energy.

This is the state-of-the-arts Agriculture Portfolio. The above reported activities, considering that farmers are mainly conservators, are expected to be considered for further expansion, although the effects of the economic crisis has lead to a general contraction that may upset current trends. There are, however, also innovators among farmers who are considering going deeper into the SC downstream. Like smart manufacturers, they may create new business models to capture profits at the end of the value chain, and to provide steady service-revenue by processing, packaging, and marketing commodities and service (Piccinini *et al.*, 2015). This would allow them to capture a larger share of income in subsequent phases of SC (HOLLAND and BRUCH, 2010). From the classification of the Growth and Share Matrix built on EU-27 figures for Agriculture in the 2000-2007 period, the composition of its Secondary production portfolio results in Fig. 1:

1 - the Food and beverages industry is the main source of cash flows, having been classified as a Cash Cow, while still having great potential as a Star. It is a safe investment, although it is growing to a lesser extent than other secondary activities of Agriculture. On the other hand, its cash flow can provide funds for further investments, such as in the areas of quality wines for direct sale, and of luxury farmhouses for rural tourism (RIZZO and GIUDICE, 2013). It also appears (Table 2) that the "Food and beverages" industry has medium-high barriers to entry but this notwithstanding, it is being invaded by other industries. The reason could be that this industry is heterogeneous and, especially in the field of experience goods, niches arise in its market. This is the case with the invasion of Agriculture and its quality wines and olive oils;

2 - Land transports, Real Estate and Other Services are Dogs that Agriculture is now reducing in its investment plan. Although these are markets with low barriers to entry and are sub-

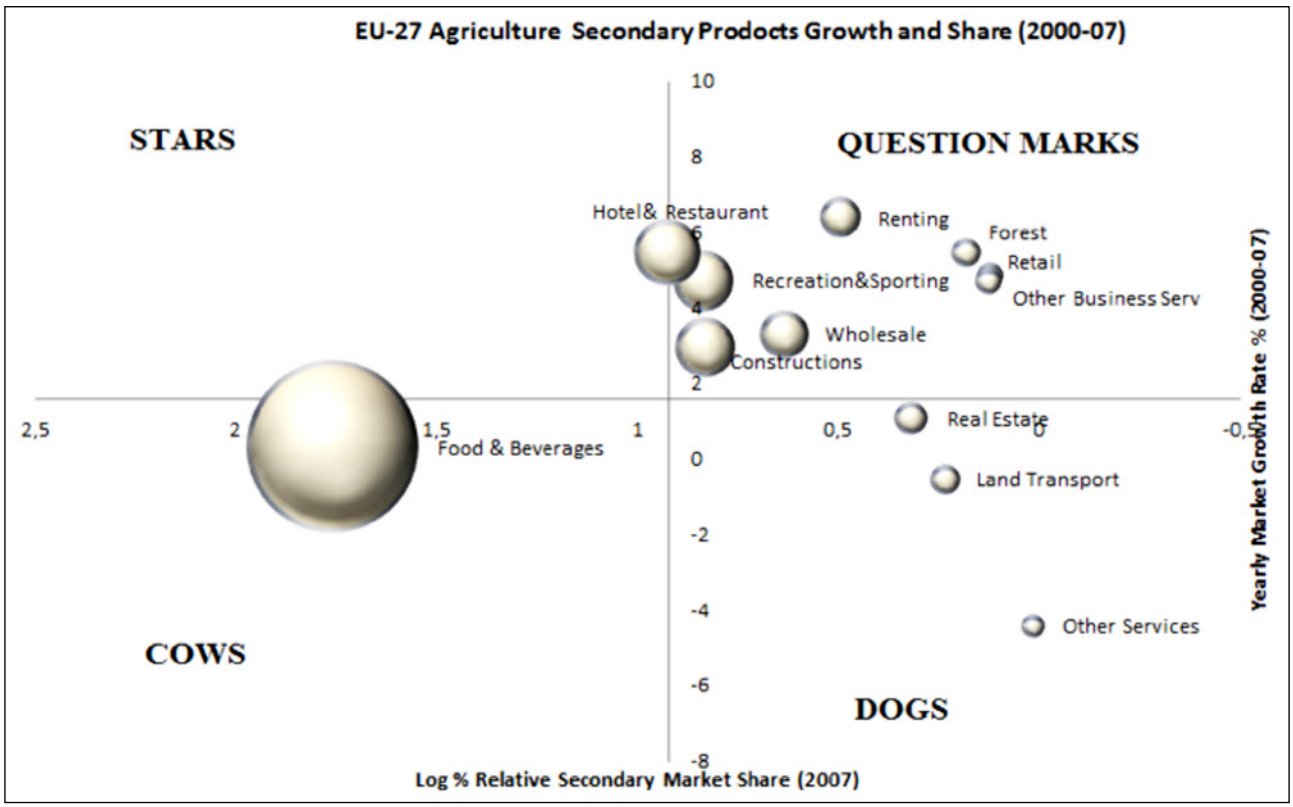


Fig. 1 - Growth and Share Matrix of Secondary Production of EU-27Agriculture (2000-07). Source: Authors' elaboration on Eurostat data.

Table 2 - Classification of the markets in which EU Agriculture performs its Secondary production.
Source: Authors' elaboration on Eurostat data.

Industries/Groups of products	Chang Entry Barriers Index	Position of Barriers in the EU Economy Ranking (Quartile)	Chang Invasion Index	Position as Invader in the EU Economy Ranking (Quartile)	Classification	
					Barriers	Capacity to Invade/ Being invaded
Products of agriculture, hunting and related services	45	I	47	I	Very High	Strong Invader
Electrical energy, gas, steam and hot water	39	I	52	I	Very High	Strong Invader
Construction work	22	II	-12	IV	High	Heavily invaded
Recreational, cultural and sporting services	21	II	33	I	Medium High	Strong Invader
Products of forestry, logging and related services	18	II	2	III	Medium High	Invaded
Food products and beverages	15	II	-4	III	Medium High	Invaded
Real estate services	0	II	-65	IV	Medium Low	Heavily invaded
Hotel and restaurant services	-3	III	-28	IV	Medium Low	Heavily invaded
Land transport; transport via pipeline services	-7	III	-4	III	Low	Invaded
Retail trade services, except of motor vehicles	-10	III	21	III	Low	Invader
Other business services	-18	III	-52	IV	Low	Heavily invaded
Wholesale trade and commission trade services	-27	IV	-3	III	Very Low	Invaded
Other services	-33	IV	-43	IV	Very Low	Heavily invaded
Renting services of machinery and equipment	-40	IV	-65	IV	Very Low	Heavily invaded

ject to being invaded (the former) or heavily invaded (the other two) by new entrants (Table 2), these activities proceed at much lower costs because they are operated by well-structured consolidated incumbents;

3 - there are no activities in Stars for which Agriculture should aim to find more Cash Cows to be milked for future investments;

4 - Question marks are numerous, namely the investments made by agriculture to diversify its portfolio of assets, which are growing at a rate higher than average and represent a real opportunity, albeit at different levels of development and portfolio share. Those which deserve to be examined for their relevance are Hotel and Restaurant (HOTREST) and Recreational, cultural and sporting services (RECREAT) which are on the borderline of being Stars as they are the most dynamic in terms of growth, and it is upon these that EU agriculture focuses its investments. HOTREST has low entrance barriers and a very high predisposition to be invaded; in consequence, Agriculture should have no problems to further expand its sphere of influence in rural tourism. RECREAT has, on the contrary, high barriers to entry and is an invader of other activities, but Agriculture has the means to circumvent these barriers since it possesses land and infrastructures to develop this type of activities (CHANG *et al.*, 2013). Among the other Question marks, namely Rental, Wholesale, Construction, Retail, Forest, and Other business services, only the first two have resisted the economic crisis to remain among the Question marks, while all the others have slipped down into the Dogs' domain. The "real estate bubble" has negatively affected some secondary activities of Agriculture because they do not have enough market share to face, through economies of scale, the challenge of the incumbent crisis. From 2007 to 2009, the number of Question marks dropped from 9 to 5, and this has frozen some agricultural expectations.

CONCLUSIONS

The managerial implications of this study are the following: firstly, it confirms the idea that entering farming's core business could be very hard for newcomers. In a stagnant market, high entry barriers and stability may influence firms, helping them to adopt a long-term viewpoint. This is the strategic element required to optimise rents deriving from firms' market share (RUMELT and WENSLEY, 1981; WERNERFELT, 1982). Secondly, it emerges that EU Agriculture has below average capabilities to engage in activities outside of its core business area. Consequently, specific measures aimed at empowering ancillary businesses should be adopted. This could be done by, for example, empowering internal operations with the implementation of pre and post-sales of in-farm services in order to familiarise and raise

awareness of new clients about its products, and deepen their knowledge of existing product-users respectively.

The final consideration is that if farms, particularly small and medium-sized, want to develop the scope of their direct sales in Retail, they must lean on Food, Hotels and Restaurants, and Recreational Activities, to create integrated projects to exploit their structures and take advantage of the conveyance of clients in proximity to wine and agro-tourism farms and to recreational estates. For example, the structures needed for the direct sale of goods and services require minimal investment and light facilities, and may become customer attractors. An empowered attractor can be an event marketing centred in rural areas, representing a fusion of the four groups: food & wine goods and services, entertainment, and retail trade. This has the advantage of concentrating the commitment of the farmers on the non-agricultural field in a short time, and of bringing a number of new potential customers. As McLuhan says in regards to these events, 'the medium is the message itself'; that is to say, the public essentially requires choral participation, and at the same time creates business (MCLUHAN, 1964).

The previous analysis indicates that farms, although following a traditional downstream model of vertical integration, may innovate along new paths in the mainstream of their secondary activities. Transactional resources such as inter-firm trade contacts (DROLI *et al.*, 2013) could help Agriculture to leverage highly lucrative markets in its portfolio such as Food, Hotels, Restaurants, and Recreational activities, to set up, plan, and organise productive business alliances (cooperation in competitive markets, partnership-based objectives, etc.).

This study does not intend to push the analysis beyond the important aspect of cash flows. Rather, it creates the necessary framework to make future investigation possible.

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ITALIAN JOURNAL OF FOOD SCIENCE -IJFS

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Acknowledgments. Acknowledgments of assistance are appropriate provided they are not related to analyses or other services performed for a fee. Financial support, thanks for assistance, artic1e number or thesis fulfilment may be included.

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