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OPINION PAPER

WHICH PROCESSING MARKERS ARE RECOMMENDED FOR MEASURING AND MONITORING THE TRANSFORMATION PATHWAYS OF MAIN COMPONENTS OF OLIVE OIL?

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ABSTRACT

Extra virgin olive oil has been increasingly investigated in scientific research for its hedonic and nutritional value. A synoptic approach on applicability of copious data available for process control appears to be a necessary, useful tool.

Based on literature data, transformation pathways of the main components of olive oil (i.e tryglicerides, phenolic compounds, volatile compounds) were described in detail in the paper. They were also attributed to olive oil chain processing, which was expressed by operating modules. A process control plan of extra virgin olive oil chain was proposed.

- Keywords: olive oil, processing markers, triglycerides, phenolic compounds, volatile compounds -

Extra virgin olive oil, which is a staple food in the Mediterranean diet, has been increasingly investigated in scientific research for its hedonic and nutritional value. A synoptic approach on applicability of copious data available for process control appears to be a necessary, useful tool.

A process control implies a selection of significant process steps (i.e., CCPs) and parameters (i.e., markers) to follow the history of quality characteristics from raw materials to end products. This entails a thorough knowledge of both positive and negative transformation phenomena, which may affect raw material components and, as a result, have positive and negative effects on the quality of end product.

Based on literature data, transformation pathways of the main components of olive oil will be described in detail in the next paragraphs. They will also be attributed to olive oil chain processing, which is expressed by operating modules. These include the following unit operations having specific homogeneous properties and optimization requirements:

• *The Growing of Olive Tree Module*. It contains all activities from selection of olive cultivar to harvesting of fully-ripe, undamaged, healthy olives.

• *The Management of Ripened Olives Module*. It contains all process operations from olive transport to washing step; these are simple operations, which however, when carried out badly, may have a negative effect on the final quality of oil.

• *The Oil Extraction Module*. This is the basic module of the production process. It contains all operations from olive crushing to olive oil separation.

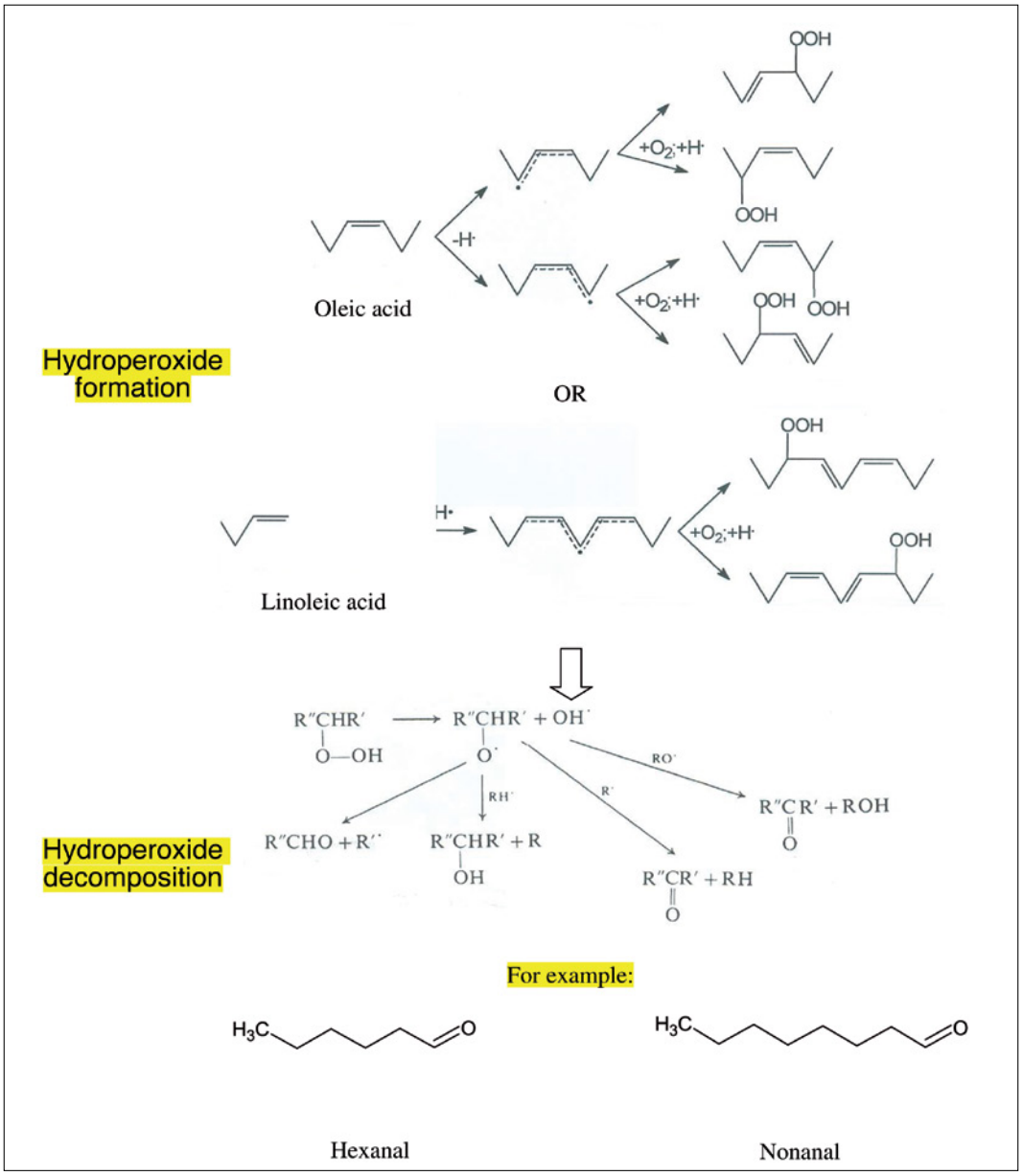


Fig. 1 - Radical oxidation of triglyceride. Adapted by FRANKEL (1991) and HAMILTON (1983).

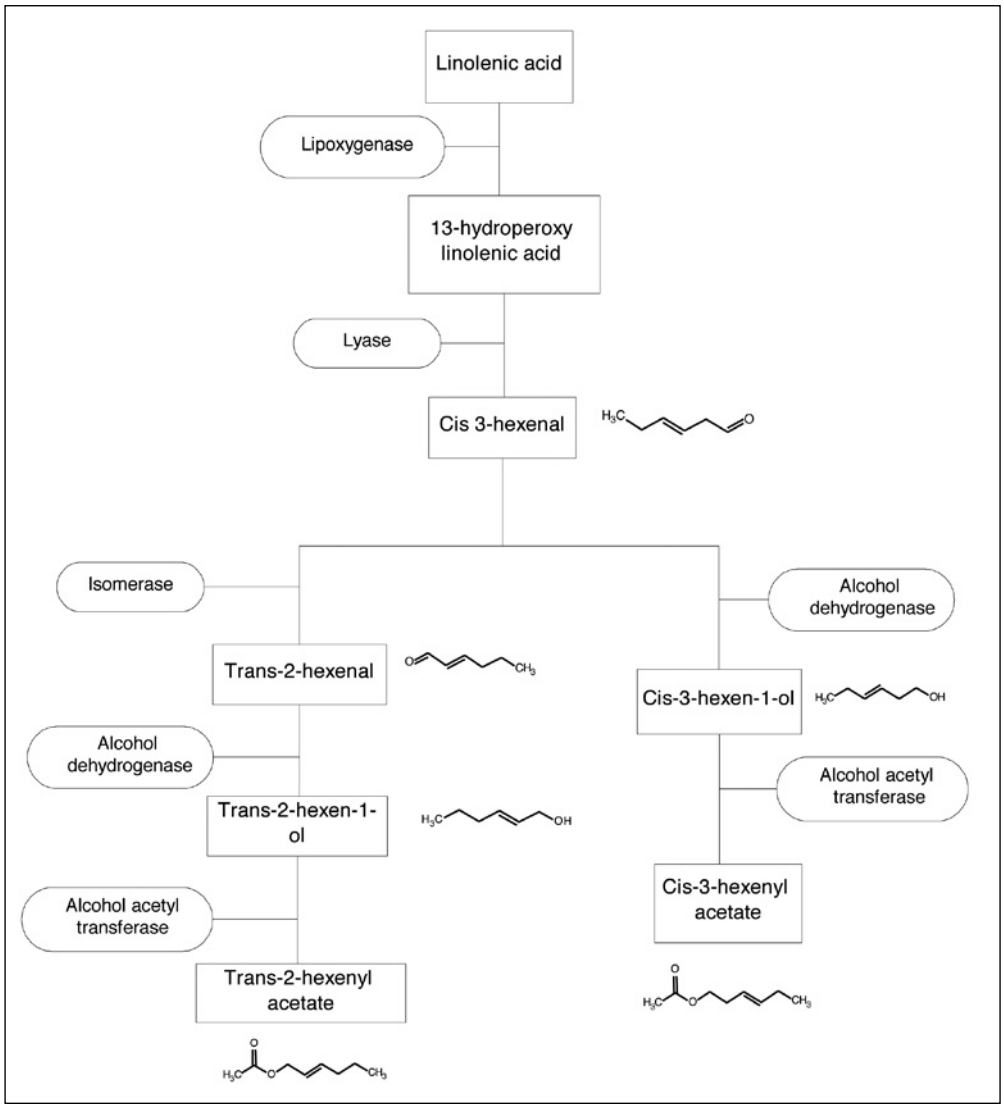


Fig. 2 - LOX pathway from linolenic acid (LnA). LOX has a preferential action on LnA compared to linoleic acid.

• *The Oil Storage and Distribution Module*. It aims at maintaining extra virgin olive oil characteristics as much unchanged as possible over time. It contains all operations from oil clarification by decanting to product distribution on the market.

TRANSFORMATION PATHWAYS OF TRIGLYCERIDES

Olive oil triglycerides are biosynthesized from the simple sugars of olives. The precursor of biosynthesis of fatty acids is acetyl-CoA, derived from catabolism of glucose and fructose (LAVEE, 1986).

Triglycerides can be degraded to free radicals and hydroperoxides by auto-oxidation and photo-oxidation (i.e., *radical oxidation*). Hydroperoxides, being very unstable, break down to alkoxy free radicals, which decompose to aldehydes, alcohols and ketones from 6 to 11 atoms of carbon (Fig. 1). These compounds are volatile and responsible for oil rancid defect; hexanal and nonanal are often considered significant compounds to contribute to the typical rancid odor for oxidized oil (APARICIO *et al.*, 2012; FRANKEL, 1991; HAMILTON, 1983).

Triglycerides can also be degraded to free fatty acids by enzymatic hydrolysis (i.e., lipase activity). Free fatty acids can either be subjected to radical oxidation or be transformed into aldehydes, alcohols and esters with 6 atoms of carbon by an enzyme oxidation, called the lipoxygenase pathway (i.e., *LOX pathway*) (ANGEROSA *et al.*, 2000). These volatile compounds from LOX pathway are responsible for pleasant sensory descriptors, such as fruity and grassy (Fig. 2).

All the above phenomena may occur at different steps along the olive oil chain. Presence of water, fruit surface scratches and olive crushing are known to favor enzymatic reactions. These are typically defined as fast reactions when optimal temperature conditions

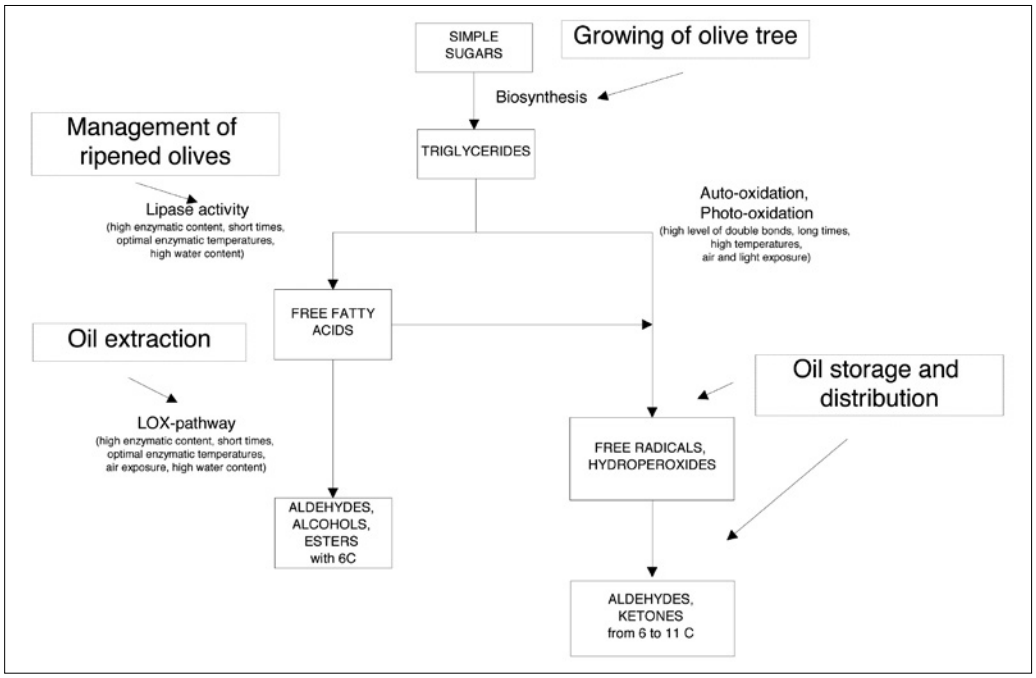


Fig. 3 - Transformation pathway of triglycerides (bracketed text explains operating conditions that favour relevant phenomena).

are available and, for oxidative reactions, also in the presence of oxygen (i.e., high redox potential values). Enzymes have both an endogenous and exogenous nature as a result of contamination by moulds, bacteria and yeasts (VICHI *et al.*, 2011).

Instead, both absence of water and long holding times at critical temperatures are known to favor non-enzymatic auto-oxidation and photo-oxidation, which are typically defined as slow reactions.

Therefore, transformation phenomena of triglycerides can be related to olive oil chain steps as follows (Fig. 3): (i) biosynthesis of triglycerides occurring during growing of olive tree module; (ii) lipase activity occurring during the management of ripened olives module; (iii) enzyme oxidation occurring during the oil extraction module; (iv) radical oxidation occurring during the oil storage and distribution module.

TRANSFORMATION PATHWAYS OF PHENOLIC COMPOUNDS

Simple sugars are also precursors of olive oil phenolic compounds. Oleuropein and ligstroside are the most important secoiridoids in olives. In the proposed biosynthetic pathway for secoiridoid production (RYAN *et al.*, 2002), simple sugars give rise to amino acids, phenylalanine and tyrosine, which in turn are changed into phenolic acids and then secoiridoids. During olive ripening the typical browning of ripe fruits occurs. This colour change is due to the decrease in clorophylls and secoiridoids and formation of anthocyanins (EL RIACHY *et al.*, 2011).

Oleuropein and ligstroside are thought to be subjected to degradation, resulting in hydrolytic and oxidative changes of both an enzymatic and non-enzymatic nature (ARTAJO *et al.*, 2007; GUTIERREZ-ROSALES *et al.*, 2010; KALUA *et al.*, 2006, SERVILI *et al.*, 2004).

Hydrolytic degradation pathway (Fig. 4) causes fast formation of aglycons (3,4-DHPEA-EA - oleuropein aglycone; p-HPEA-EA - ligstroside aglycone), as a result of the loss of a sugar molecule, which can be caused by β-glucosidase activity. Aglycons thus obtained can undergo isomerization, which involves opening of the secoiridoidic ring, followed by rearrangement into open dialdehydic forms. Dialdehydic forms in turn decarboxylate into respective aglycons (3,4-DHPEA-EDA - dialdehydic form of decarboximethyl oleuropein aglycone; p-HPEA-EDA - dialdehydic form of decarboximethyl ligstroside aglycone). Finally hydroxytyrosol (3,4-DHPEA) and tyrosol (p-HPEA) compounds are slowly formed by hydrolysis of the ester linkage.

Literature data show relationships both between 3,4-DHPEA-EA and bitter intensity and between p-HPEA-EDA and pungency intensity. Oleuropein, p-HPEA-EDA (i.e., oleocanthal), 3,4-DHPEA and p-HPEA compounds have been demonstrated to have human healthy effects.

Oxidative degradation of secoiridoids may be both an enzymatic degradation pathway and a non-enzymatic degradation pathway. In the former pathway, polyphenoloxidases (PPO) and peroxidases (POD) in olive paste catalyze, in the presence of oxygen and high water content, the oxidation of phenolic compounds to corresponding quinones; evidence for this oxidation

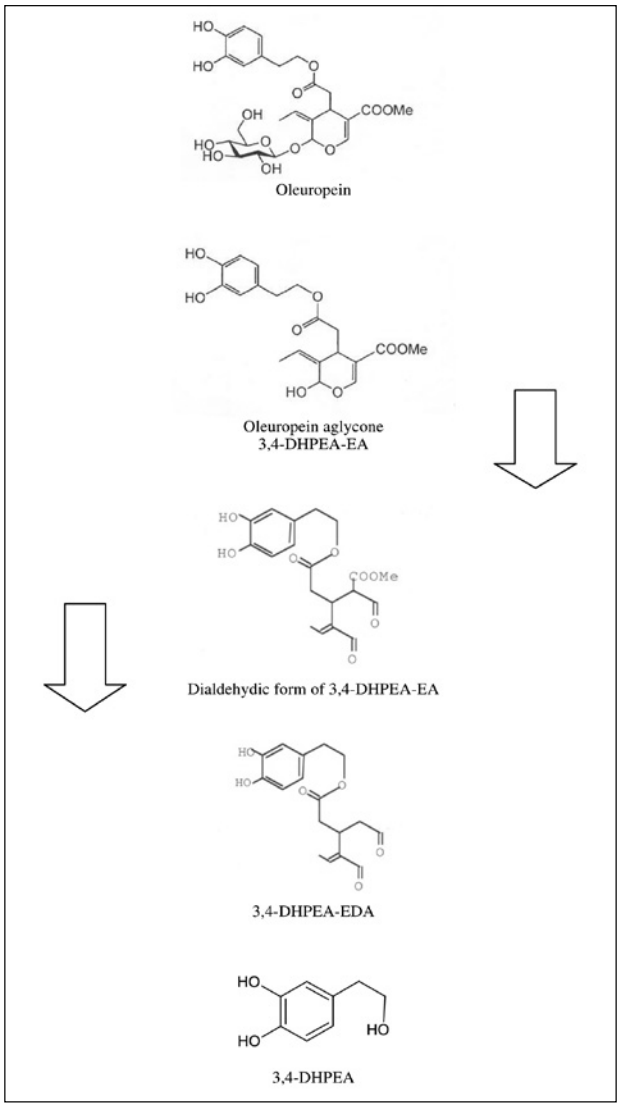


Fig. 4 - Hydrolytic degradation pathway of oleuropein.

is provided by browning of olive paste being processed. In the latter pathway, which is connected to termination reactions of radical auto-oxidation of triglycerides to peroxides and derivatives, the release of hydrogen atoms by phenolic compounds can inhibit the formation of hydroperoxide radicals.

The above-mentioned phenomena cause a degradation of phenolic compounds and can bring about changes in both intensity of sensory descriptors such as bitter and pungent and degree of antioxidant power of oil, resulting in decreased shelf-life and nutritional value of product. An exception is represented by β-glucosidase activity that increases the amount of phenolic compounds in olive oil, as aglicons are more soluble in oil than oleuropein and ligstroside.

Phenomena on secoiridoids have been shown to occur at different steps of extra virgin olive oil chain (Fig. 5).

Both biosynthesis of secoiridoids and their first enzymatic hydrolysis to form aglicons occur during growing of olive tree module. A remarkable presence of 3,4-DHPEA and p-HPEA also in ripe olives (VINHA *et al.*, 2005) should be regarded as a result of biosynthetic pathway of secoiridoids as well as formation of anthocyanins (EL RIACHY *et al.*, 2011).

During oil extraction module, especially during olive crushing and malaxation, enzymatic degradation prevails, as well as the first steps of hydrolytic degradation, leading to the formation of dialdehydic forms of decarboxymethyl aglycons. Instead, it is non-enzymatic oxidative degradation as well as 3,4-DHPEA and p-HPEA forming hydrolytic degradation that prevail during oil storage and distribution module. It should

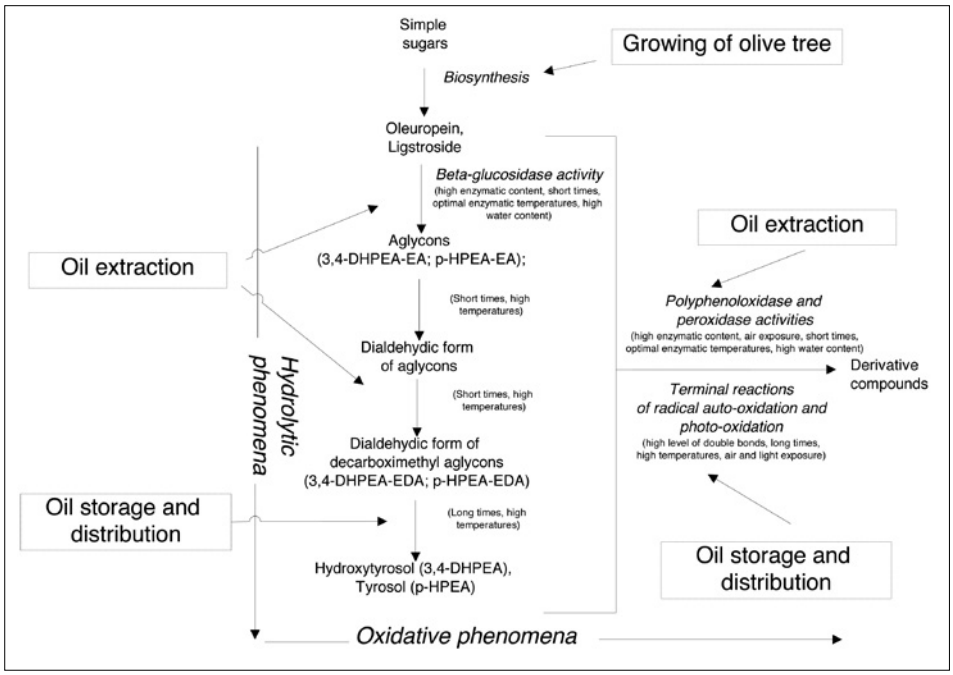


Fig. 5 - Transformation pathway of secoiridoids (bracketed text explains operating conditions that favour relevant phenomena).

Table 1 - Correlation between volatile compounds and sensory attributes.

Volatile compounds	Sensory attributes	Involved phenomena
Hexan-1-ol 1-Penten-3-ol cis-2-Penten-1-ol cis-3-Hexen-1-ol *trans*-2-Hexen-1-ol Hexanal cis-3-Hexenal *trans*-2-Hexenal 2,4-Hexadienal 1-Penten-3-one Hexyl acetate cis-3-Hexenyl acetate *trans*-2-Hexenyl acetate	Fruity and other positive attributes	LOX pathway
Butyric acid Butan-2-ol Butyl acetate Ethyl butirate Ethyl propionate Methyl propionate	Negative attribute: Fusty or muddy sediment	Microbial anaerobic fermentation
Benzene-ethanol Phenol 4-Ethylphenol Guaiacol 4-Ethyl guaiacol cis-1-Otten-3-ol Pentanal Octane	Negative attribute: Musty or humid	Moulds and yeasts development
Ethyl acetate Methyl acetate Cis-2-esenil acetate	Negative attribute: Winey-vinegary or acid-sour	*Lactobacillus* and yeasts development
Benzaldehyde Pentanoic acid 1-Pentanol *trans*-2-Pentenal Hexanoic acid Hexanal Heptane 1-Heptanol 2-Heptanol Heptanal 2,4-Heptadienal *trans*-2-Heptenal 6-Methyl-5-epten-2-one 2-Heptanone Octanoic acid 1-Octanol cis-1-Octen-3-ol *trans*-2-Octanal Octanal 2-Octanone 1-Octen-3-one 1-Nonanol Nonanal 2,4-Nonadienal *trans*-2-Nonenal *trans*-2-Decenal 2,4-Decadienal	Negative attribute: Rancid	Radical oxidation of triglycerides

be noted that enzymatic activities have both an endogenous and exogenous nature as a result of contamination by moulds, bacteria and yeasts (VICHI *et al.*, 2011).

EXTRA VIRGIN OLIVE OIL VOLATILE COMPOUNDS

Even though they are minor components of extra virgin olive oil, volatile compounds have a critical role on liking and legal conformity of product. They are so numerous that no clear pathways of biosynthesis and transformation have been so far evidenced. However, volatile compounds can be correlated with common sensory attributes, both positive and negative (DI GIACINTO *et al.*, 2010; MORALES *et al.*, 2005), as shown in Table 1.

Taking into consideration the involved phenomena reported in Table 1, fusty, musty and winey-vinegary defects have been shown to be influenced by operating conditions performed during both the management of ripened olives module and the oil extraction module. Microbial contamination and activity are very important to develop the above negative attributes. Instead, rancid negative attribute may significantly occur during the oil storage and distribution module. Fruity attribute is expressed by LOX pathway, it is then influenced by the oil extraction module conditions.

All above-mentioned phenomena of synthesis and transformation are also limited by component mass transfer to oil matrix during processing.

Triglycerides content depends on the operating conditions for olive oil extraction. Malaxation has to create a continuous mass of oil in the olive paste; in order to disrupt the interfacial films, olive paste has to be mixed modifying the dispersion degree of oil droplets and promoting the coalescence phenomena. Separation, for example by “decanter” centrifugation, has to maximize olive oil yield separation, minimizing retention times to maintain the positive effects of malaxation on olive oil yield.

Secoiridoids content depends on their solubility in oil and water. They are amphipathic molecules, but their polarity makes them more soluble in water than in oil; therefore, their concentration in oil is always very low. However, solubility of secoiridoids may change as a result of their transformation pathway. Elimination of glucose by secoiridoids determines a change of polarity, and the relevant aglycones are more soluble in oil than in water. Therefore, malaxation and separation are critical conditions; they have to aid mass transfer of secoiridoids to oil and to minimize secoiridoids removal with vegetation water.

Table 2 - Process control of extra virgin olive oil chain.

What? Monitoring phenomena	Where? Critical Control Points (CCPs)	How? Basic level control markers	How? High level control markers	Why? Effects on EVOO quality
Biosynthesis of TGs	Olive harvesting	Visual inspection for olive ripening indices	Olive oil content	Yield
Enzymatic hydrolysis of TGs	Olive storage	Visual inspection for olive sanitary conditions	Olive mould count	Acidity level Shelf-life stability
	Olive oil separation	Olive oil acidity		
LOX-pathway of TGs	Olive oil separation	Olive oil taste	Sum of C6 volatile compounds content from C18:3 free fatty acid	Fruity level
Radical oxidation of TGs	Olive oil storage	Olive oil taste Peroxide value UV spectroscopic indices	Sum of hexanal and nonanal volatile compounds content	Rancid level Peroxide value level UV spectroscopic indices level Antioxidant power
Mass transfer of TGs	Olive paste malaxation	Visual inspection for olive oil coalescence	Microscopic or rheological measurements of olive oil coalescence	Yield
	Olive oil separation	Visual inspection for efficacy of separation	Material balances	
Biosynthesis of PhCs	Olive harvesting	Visual inspection for olive ripening indices	Total phenolic compounds content Oleuropein content	Bitter/Pungency level Antioxidant power Shelf-life stability
Hydrolytic phenomena of PhCs	Olive oil separation	-	3,4-DHPEA-EDA content	Antioxidant power Shelf-life stability
	Olive oil storage	-	3,4-DHPEA content	
Oxidative phenomena of PhCs	Olive paste malaxation	Olive paste color	3,4-DHPEA-EDA content	Bitter/Pungency level Antioxidant power Shelf-life stability
	Olive oil storage	Total phenolic compounds content		
Mass transfer of PhCs	Olive paste malaxation	-	Total phenolic compounds content 3,4-DHPEA-EDA content	Bitter/Pungency level Antioxidant power Shelf-life stability
	Olive oil separation	Olive oil taste Total phenolic compounds content	3,4-DHPEA-EDA content Material balances	
Microbial contamination and activity	Olive storage	Visual inspection for olive sanitary conditions	Olive mould and yeast count	Fusty, Musty and Winey-vinegary level
	Olive oil separation	Olive oil taste	Sum of volatile compounds correlated with negative attributes	

TGs - Triglycerides; PhCs - Secoiridoids; EVOO - Extra Virgin Olive Oil.

CCPS AND MARKERS OF EXTRA VIRGIN OLIVE OIL PROCESS CONTROL

Process control is focused on monitoring, which is the act of conducting a planned sequence of measurements of parameters to assess if process is under control.

Monitoring of extra virgin olive oil production may be planned at two levels.

The first level aims at controlling planned operating conditions (i.e., residence times, temperatures, rotational speeds, clarity levels, etc.). It differs according to the equipment and plants used, and general indications cannot be given on this level of monitoring.

The second level aims at controlling the effects of operating conditions on product during processing. It is not dependent upon machines used, and therefore, monitoring may be generally performed. Table 2 shows an example of this approach.

The above-mentioned transformation phenomena of the main components of olive oil are planned to be monitored by critical steps of process (i.e., CCPs). Some significant parameters (i.e., markers) are chosen to be measured in olive oil samples. Two levels of control are identified: a basic and a high level. They require increasing analytical work, and, however, allow for increasing efficacy in terms of control. High

level control markers are a more direct expression of monitoring phenomena than basic level control markers.

Effects of monitored phenomena on extra virgin olive oil quality are shown in the last column in Table 2.

Basic level control includes chemical and sensory markers, which are commonly measured to control conformity of both process extraction and extra virgin olive oil. Some table cells are blank, as the relevant simple markers have not yet been identified in the literature.

With reference to high level control, some overall markers are chosen for LOX-pathway, radical oxidation of triglycerides and activities of spoilage microorganisms such as "Sum of C6 volatile compounds content from free linolenic acid", "Sum of hexanal and nonanal volatile compounds content" and "Sum of volatile compounds correlated with negative attributes", respectively.

3,4-DHPEA-EDA may be considered a direct marker of both extraction yield and integrity of phenolic compounds during olive oil processing; whereas 3,4-DHPEA/hydroxytyrosol may be considered a marker of degradation of phenolic compounds during olive oil storage.

Microbial counts are also included to monitor mould and yeast contamination in olives and oil.

Material balances are finally suggested to measure efficacy of component mass transfer to oil during malaxation and separation.

CONCLUSIONS

The following conclusive remarks can be drawn from the foregoing observations.

Extra virgin olive oil can be regarded not just as a product obtained from simple mechanical extraction from olives; rather, it should be considered as a result of complex changes in fruit components. These changes affect, on the one hand, both free and esterified fatty acids, and on the other hand, secoiridoid phenolic compounds, resulting in formation of derivatives, which impact qualitative characteristics for both sensory acceptability and nutritional value of extra virgin olive oil.

Health claims related to polyphenols in olive oil (Anonymous, 2011) attribute positive effects (i.e., antioxidant properties and positive effect on lipid metabolism) on human health, when the following conditions of use are applied: "*20 g of an olive oil with a polyphenol content of 200 mg/kg / Min 2 mg per day of hydroxytyrosol*". From a nutritional point of view, a reference to hydroxytyrosol appears to be appropriate, as it is one of the most powerful antioxidants. This reference, however, appears inappropriate from a production point of view, as hydroxytyrosol (3,4 DHPEA) is a direct marker of hydrolytic degradation of phenol compounds.

Microorganisms take on a significant role in transformation pathways of extra virgin olive oil. Mould, bacteria and yeast contamination is especially associated with sensory defects and enzymatic degradation activities.

Although transformation pathways of the main components of olive oil during processing are known, no data on relevant kinetics (i.e., pseudo-n order reactions, K_0 and E_a) are reported in the literature. It would be useful and recommended to collect a large number of data on this subject. This would avoid incomplete production recommendations such as the customary rule that temperature of malaxation has not to exceed 27°C. Nothing confirms that 27°C is a temperature tied to some particular phenomenon; in addition, indication of temperature without indication of time is incomplete and misleading recommendation.

In view of numerous complex transformations that occur in olive oil components during the extraction process, the current approach to optimize operating conditions of oil mill is still based on the "*trial and error*" principle, using a limited number of results from numerous literature data. On the other hand, obtaining useful information of wide application on the effect of either olive or extraction processing on the quality of extra virgin olive oil may appear to be difficult and misleading. This may derive from the fact that most experimental designs have been developed on the basis of industrial oil mills and mini oil mill plants, which are characterized by poor reproducibility and laborious process control. An experimental approach based on lab-scale processing under controlled conditions (Table 2) appears a convenient alternative.

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PAPER

ENTEROCIN PRODUCTION BY *ENTEROCOCCUS FAECIUM* STRAINS ISOLATED FROM GREEK SPONTANEOUSLY FERMENTED SAUSAGES

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ABSTRACT

The aim of this study was to assess enterocin production by 22 *Enterococcus faecium* strains previously isolated from spontaneously fermented sausages. Enterocins production exhibited similar kinetics with most other bacteriocins produced by lactic acid bacteria and a rather limited antimicrobial spectrum. Occurrence of multiple genes encoding for different enterocins seemed to be a common feature. The enterocins were heat stable and the antimicrobial activity was retained over a wide range of pH values and chemicals, some of which are frequently used in fermented meat products.

- Keywords: *Enterococcus faecium*, enterocins, spontaneously fermented sausages -

INTRODUCTION

Bacteriocinogenic enterococci are widespread in nature; they have been isolated from a wide variety of both animal- and plant- derived fermented material (RIVAS *et al.*, 2012; STROMPFOVA *et al.*, 2008). Over the years, many peptide bacteriocins have been genetically characterized and purified, most of them obtained from *Enterococcus faecium* and *E. faecalis* (NES *et al.*, 2007).

The bacteriocinogenic potential of enterococci isolated from spontaneously fermented meat products has been studied to some extent and enterocins A, B and P have been described (AYMERICH *et al.*, 1996; CASAUS *et al.*, 1997; CINTAS *et al.*, 1997). Moreover, *in vitro* and *in situ* production, the effect of various abiotic factors on enterocin activity, as well as the effectiveness of enterocin-producing strains or their enterocins to control growth of *Listeria monocytogenes* in experimentally contaminated fermented sausages, has also been studied (LAUKOVA *et al.*, 1999; CALLEWAERT *et al.*, 2000; AYMERICH *et al.*, 2000; SABIA *et al.*, 2003; ANANOU *et al.*, 2005; DROSINOS *et al.*, 2008).

Spontaneously fermented Greek sausages have been studied to some extend (NYCHAS *et al.*, 2012). It has been exhibited that enterococcal population ranged between 10^4 and 10^6 CFU g^{-1}. The enterococcal ecosystem has been studied in-depth by PARAMITHIOTIS *et al.* (2008). In the latter study, wide strain diversity was revealed and 108 *E. faecium* strains, isolated from 3 batches of spontaneously fermented sausages made in the same production plant were clustered into 21 different pulsotypes. Furthermore, 42 of those isolates classified in 13 different pulsotypes, were able to produce antimicrobial compounds of protein nature that could inhibit growth of *L. monocytogenes* strain NCTC 10527.

The purpose of the present study was to assess the diversity of the enterocins produced by 22 *E. faecium* strains representing the aforementioned 13 different pulsotypes. Therefore, presence of structural genes encoding enterocin production, enterocin production kinetics, antimicrobial spectrum along with the effect of temperature, pH and selected chemicals on enterocin activity has been studied.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The *Enterococcus faecium* strains used throughout this study along with all indicator strains, except for *Listeria monocytogenes* strains NCTC 10527 and ATCC 19115, *Escherichia coli* strains NCTC 12079 and ATCC 25922 as well as *Staphylococcus aureus* strains ATCC 6538 and NCBF 1499 were previously isolated from spontaneously fermented sausages (DROSINOS *et al.*, 2005; 2007) and are listed in Table 1. The 22 enterococcal strains were selected to represent all different bacteriocin-producing pulsotypes obtained by PARAMITHIOTIS *et al.* (2008). All strains were stored at -80°C in Brain Heart Infusion (BHI) broth (LAB M, Bury, Lancashire, UK) or, in the case of lactic acid bacteria, in de Mann Rogosa Sharpe (MRS) broth (LAB M) supplemented with 20% (v/v) glycerol (Sigma Chem-

Table 1 - Inhibitory spectrum of the *E. faecium* strains under study.

	***Enterococcus faecium* strains**																					
Indicator strains	**20001**	**20002**	**20005**	**20006**	**20007**	**20011**	**20015**	**20020**	**20023**	**20038**	**20039**	**20040**	**20044**	**20045**	**20049**	**20074**	**20081**	**20083**	**20093**	**20098**	**20101**	**20102**
Listeria monocytogenes (13 strains)	1 [1,2]	1[3]	2[4]	-	2[4]	-	-	2[4]	-	-	1[2]	-	-	2[4]	-	1[3]	1[2]	1[2]	-	-	3[5]	1[2]
L. monocytogenes NCTC 10527	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactobacillus plantarum (20 strains)	-	-	2[6]	-	-	-	2[6]	2[6]	-	-	-	-	-	2[6]	-	-	2[6]	-	-	-	-	-

No inhibition was observed when *L. monocytogenes* ATCC 19115; *Salmonella* sp. (14 strains); *E. faecium* (22 strains); *Lactobacillus curvatus* (20 strains); *Lb. sakei* (20 strains); *Staphylococcus saprophyticus* (14 strains); *St. simulans* (16 strains); *St. xylosus* (20 strains); *St. aureus* (7 strains); *St. aureus* ATCC 6538; *St. aureus* NCBF 1499; *Escherichia coli* NCTC 12079 and *E. coli* ATCC 25922 were used as indicator microorganisms.

[1]number of indicator strains whose growth was inhibited; [2]*L. monocytogenes* strain LQC 15142; [3]*L. monocytogenes* strain LQC 15254; [4]*L. monocytogenes* strains LQC 15142 and LQC 15254; [5]*L. monocytogenes* strains LQC 15142, LQC 15253 and LQC 15254; [6]*Lb. plantarum* strains LQC 849 and LQC 872.

+: antimicrobial activity;

-: no antimicrobial activity.

ical Co, St Louis, Missouri, USA). Before experimental use strains were subcultured twice in the appropriate media.

Antimicrobial spectrum

The antimicrobial spectrum was assessed using the well diffusion assay according to PARAMITHIOTIS *et al.* (2006). Plates were incubated at 37°C (30°C when LAB were used as indicator strains) for 48 h and examined for clear zones of inhibition. All assays were performed in triplicate.

Bacteriocin production kinetics

Each bacteriocin producing strain was inoculated in BHI broth (pH 7.3) at a level of 10^4 cfu mL^{-1} and incubated at 37°C. Viable cell counts were made by plating serial dilutions onto BHI agar, pH value was recorded and bacteriocin activity was determined using the critical dilution method after 1, 3, 5, 7, 9, 12, 18, 24, 36, and 48 h. One arbitrary unit (AU) was defined as the reciprocal of the highest dilution showing a clear inhibition zone multiplied by 100 to obtain AU mL^{-1}. *L. monocytogenes* strain NCTC 10527 was used as indicator. All assays were performed in triplicate. Average growth and pH reduction curves are presented, representing the respective of all *E. faecium* strains under study.

Detection of structural genes encoding enterocin production

DNA was isolated according to PARAMITHIOTIS *et al.* (2010). PCR amplification of the structural genes encoding for enterocins A, B, P, L50A and L50B was performed as described by ALMEIDA *et al.* (2011). PCR products were visualized by electrophoresis on 8% polyacrylamide-Tris-acetate-EDTA gel and ethidium bromide staining. All assays were performed in triplicate.

Effect of pH, selected chemicals and temperature on bacteriocin activity

Bacteriocin producing strains were grown in BHI broth at 37°C for 24 hours. The effect of pH, selected chemicals and temperature on bacteriocin activity was assessed as previously described (TODOROV *et al.*, 2011). In brief, the effect of pH was assessed by adjusting the cell-free supernatants of all strains from pH 2.0 to 12.0 (at increments of one pH unit) with sterile 1.0 N NaOH or 1.0 N HCl. After incubation at 30°C for 2 hours, the pH of the samples was readjusted to 6.5 and residual bacteriocin activity was assessed by the well diffusion assay. The effect of selected chemicals (sodium dodecyl sulphate, Tween 80, Triton X-100, Urea, NaCl, Na-EDTA, ascorbic acid and sodium nitrate) was assessed by adding 1% w/v (final concentration) to cell-free supernatant of all strains. Residual bacteriocin activity was assessed after 5h incubation at 37°C by well diffusion assay. The effect of temperature was assessed by incubating cell-free supernatants of all strains at 4°, 20°, 30°, 37°, 45°, 60°, 80°, and 100°C. Residual bacteriocin activity was assessed after 30, 60, 90 and 120 minutes by the well diffusion assay. All assays were performed in triplicate.

RESULTS

In Table 1 the inhibitory spectrum of the *Enterococcus faecium* strains under study is shown. All strains exhibited antibacterial activity against at least one *Listeria monocytogenes* strain, and five against two *Lactobacillus plantarum* strains.

In Fig. 1, growth of the *E. faecium* strains under study, reduction of the pH value and antimicrobial activity is presented. Regarding the latter, two major groups have been identified; namely groups 1 and 2. Strains belonging to each group are designated in Table 2. In both groups production started during early exponential growth and maximum production (2.9 and 4.2 log AU mL^{-1} for groups 1 and 2, respectively) was recorded at the beginning of the stationary phase. Then a decrease in the bacteriocin activity was observed until minimum value at the end of the incubation period.

Occurrence of structural enterocin genes among the strains under study is shown in Table 2. In most of the strains, i.e. 14 out of 22, multiple genes were detected; in two strains only one gene and in six strains no genes were detected.

The antimicrobial activity of the supernatant withstood incubation at all examined temperatures, even after 120 min. In Table 3 the effect of pH and selected chemicals on bacteriocin activity is shown. Regarding the former, differences

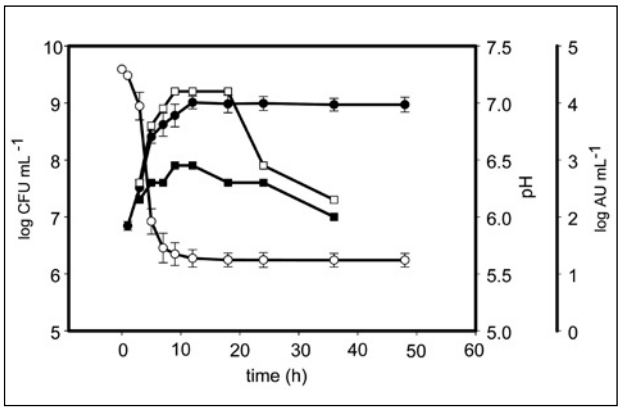


Fig. 1 - Viable cell count of *E. faecium* strains under study (●:cfu/mL), pH reduction (○), and antimicrobial activity (■:group 1; □:group 2).

Table 2 - Occurrence of structural enterocin genes among *E. faecium* strains under study.

E. faecium strains	Enterocins				
	A	B	P	L50A	L50B
20001[1]	-	-	-	-	-
20002[2]	-	-	-	-	-
20005[1]	+	-	+	-	-
20006[1]	+	-	+	-	-
20007[2]	+	-	+	-	-
20011[1]	+	+	+	-	-
20015[1]	+	-	+	-	-
20020[2]	-	-	-	-	-
20023[2]	+	+	-	-	-
20038[1]	+	-	-	+	+
20039[1]	-	-	+	-	-
20040[1]	+	+	+	-	-
20044[2]	+	+	-	+	+
20045[1]	-	-	-	-	-
20049[2]	+	+	-	-	-
20074[2]	+	+	-	+	+
20081[1]	+	-	+	-	-
20083[1]	-	-	-	-	-
20093[2]	+	+	+	-	-
20098[1]	+	-	-	+	+
20101[2]	-	-	-	-	-
20102[1]	-	-	+	-	-

[1, 2]: strains belonging to groups 1 and 2, respectively, according to their bacteriocin production kinetics;
+: presence of the respective structural enterocin gene;
-: absence of the respective structural enterocin gene.

were observed as far as pH 3 and 12 were concerned. After exposure to pH 3, antimicrobial activity was detected in only 2 strains. On the contrary, the antimicrobial activity of 6 strains was negatively affected after exposure to pH 12. Treatment with SDS, urea, NaEDTA, ascorbic acid and sodium nitrate had no effect on bacteriocin activity. On the contrary, no antimicrobial activity was detected in two, ten and three strains after treatment with Tween-80, Triton X-100 and NaCl, respectively (Table 3).

DISCUSSION

Enterococci in general and *E. faecium* in particular, are well known bacteriocin producers. Their inhibitory spectrum is characterized by their particular effectiveness on *L. monocytogenes*, both *in vitro* and *in situ*, due to their phylogenetic proximity (DEVRIESE and POT, 1995; HUGAS *et al.*, 2003) and extends over a wide range of Gram-positive microorganisms including listeriae, clostridia, bacilli, lactobacilli, lactococci, pediococci, enterococci and bifidobacteria (RIVAS *et al.*, 2012; DE VUYST *et al.*, 2003; FRANZ *et al.*, 1996; GIRAFFA 1995; HU *et al.*, 2010). In the present study, a rather limited antimicrobial spectrum has been revealed, most probably due to the common isolation source of producer and the majority of the indicator strains.

Bacteriocin production exhibited similar ki-

Table 3 - Effect of pH and selected chemicals on bacteriocin activity.

E. faecium strains	pH				Chemicals		
	2	3	4-11	12	Tween-80	Triton X-100	NaCl
20001	-	-	+	+	-	-	+
20002	-	-	+	+	-	+	+
20005	-	-	+	-	+	-	-
20006	-	-	+	-	+	-	-
20007	-	-	+	+	+	+	+
20011	-	-	+	+	+	+	+
20015	-	-	+	+	+	+	+
20020	-	-	+	+	+	-	+
20023	-	+	+	+	+	+	+
20038	-	-	+	+	+	+	+
20039	-	-	+	-	+	-	+
20040	-	-	+	+	+	+	+
20044	-	-	+	+	+	+	+
20045	-	-	+	-	+	-	-
20049	-	-	+	+	+	+	+
20074	-	-	+	-	+	-	+
20081	-	-	+	+	+	-	+
20083	-	-	+	+	+	+	+
20093	-	-	+	+	+	+	+
20098	-	-	+	+	+	+	+
20101	-	+	+	+	+	-	+
20102	-	-	+	-	+	-	+

+: no effect on bacteriocin activity;
-: no bacteriocin activity was detected.

netics not only with the ones produced by other *E. faecium* strains (DU TOIT *et al.*, 2000; KANG and LEE, 2005; TORRI TARELLI *et al.*, 1994; FRANZ *et al.*, 1996; DO NASCIMENTO *et al.*, 2010) but also with most other bacteriocins produced by lactic acid bacteria (HENG *et al.*, 2007).

Occurrence of multiple genes encoding for different bacteriocins seems to be a common feature in enterococci. Presence of as many as five different genes has been described (ALMEIDA *et al.*, 2011; DE VUYST *et al.*, 2003). This was also the case in the present study; most of the strains contained more than one structural enterocin genes. The inability to detect any of the known structural genes in six strains might be due to the existence of still undescribed ones.

Enterocins produced by *E. faecium* have been reported to be heat stable (RIVAS *et al.*, 2012; KANG and LEE, 2005; DE KWAADSTENIET *et al.*, 2006) and retain activity even after heating at 121°C for 15 min (DU TOIT *et al.*, 2000). This was also the case in the present study; antimicrobial activity was retained even after heating at 100°C for 120 min. As far as the effect of pH on enterocin activity was concerned, a remarkable stability was observed over a range of pH values from 3 to 12 concurring with the ones reported in the literature (DU TOIT *et al.*, 2000; DE KWAADSTENIET *et al.*, 2006). As far as the effect of selected chemicals on enterocin activity was concerned, a lack in the literature is evident, at least regarding the ones used in the development of fermented meat products.

Overviewing the results obtained from this study, enterocin production exhibited similar kinetics with most other bacteriocins produced by lactic acid bacteria and a rather limited antimicrobial spectrum. Occurrence of multiple genes encoding for different enterocins seemed to be a common feature. Finally, heat stability and maintenance of the antimicrobial activity over a wide range of pH values and chemicals, some of which are frequently used in fermented meat products, have been documented.

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PAPER

DEVELOPMENT OF NEW TYROSINASE TYPE TIME TEMPERATURE INDICATOR

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ABSTRACT

A new enzymatic time temperature indicator based on the reaction catalyzed by tyrosinase was developed. Time temperature indicators can be used as quality indicator of foods if response time of the indicator equals the shelf life and the spoilage process of the product. Three tyrosinase indicators were developed in three different L-tyrosine concentrations and stored at temperatures of 5°, 10°, 15° and 25°C. Color changes of indicators were used as response parameter during storage and their activation energies were determined as 123, 52 and 49 kJ/mol for the L-tyrosine concentrations of 0.1, 0.5 and 1 mM, respectively.

- Keywords: color, tyrosinase, time temperature indicator, activation energy, cold chain -

INTRODUCTION

Time temperature indicator (TTI) can be defined as a simple device that can show an easily measurable, a time-temperature dependent change that reflects the full or partial temperature history of a food product (TAOUKIS and LABUZA, 1989a). The principle of TTI operation is a mechanical, chemical, electrochemical, enzymatic or microbiological irreversible change usually expressed as a visible response, in the form of a mechanical deformation, color development or color movement (LABUZA and FU, 1995; SHIMONI *et al.*, 2001; KREYENSCHMIDT *et al.*, 2010; WELLS and SINGH, 1988; TSOKA *et al.,* 1998). The rate of change is temperature dependent, increasing at higher temperatures. The visible response thus gives a cumulative indication of the storage conditions that the TTI has been exposed to. The extent to which this response corresponds to a real time-temperature history depends on the type of the indicator and the physicochemical principles of its operation.

The temperature variations in a food product can lead to changes in product safety and quality (KOUTSOUMANIS *et al.*, 2006). There are two types of temperature indicators; the simple critical temperature indicators and the time-temperature indicators (PAULT, 1995; AHVENAINEN and HURME, 1997). Temperature indicators show whether products have been heated above or cooled below a reference (critical) temperature, warning consumers about the potential survival of pathogenic micro-organisms and protein denaturation during, for example, freezing or defrosting processes. To determine the changes in the shelf life of the food product, the second type of indicator, a TTI, should be employed (PAULT, 1995; KREYENSCHMIDT *et al.*, 2010). These indicators display a continuous temperature-dependent response of the food product. The changes shown by TTI can easily be measured, and have been proven to give consistent responses under the same temperature conditions and when not under the influence of light, humidity, and contaminants.

The quality of refrigerated foods is strongly dependent on temperature exposure history from production to consumption stages. If temperature abuse occurs during transport or marketing, the customer has no way of knowing the product is still usable or not even if the best before date is good. TTI's can be used as an indicative and/or effective alternative to the open dating (LABUZA and FU, 1995; SHIMONI *et al.*, 2001). Successful application of a TTI depends upon many factors, such as the knowledge of the food product, the type of monitoring required and the potential benefits as well as relative costs and reliability of the system at the temperatures to be encountered (WELLS and SINGH, 1988; TSOKA *et al.*, 1998). The reliability for TTI applications includes its variability in responses within the temperature range encountered, the confidence on the determined kinetic parameters and the difference between activation energy of food with that of TTI (TAOUKIS *et al.*,1991).

In this study, new enzyme based TTI gels were developed by entrapment of tyrosinase enzyme into acrylamide/bisacrylamide gel. Enzyme immobilization is the most promising technology that would be incorporated into food system. Entrapment is often the most desirable method because results in high recovery of enzyme activity after immobilization matrix, and protection from the microbial degradation (CABRAL and KENNEDY, 1993).

The objective of this study was to develop a new tyrosinase type time temperature indicator to monitor quality changes of foods during over the entire supply chain. This new enzymatic time temperature indicator was based on the tyrosinase catalyzed oxidation of L-tyrosine. Color change was taken as response parameter for TTI development. To check the usability of TTI for shelf life monitoring of foods, activation energies of TTI color changes were determined and evaluated for TTI development.

MATERIALS AND METHODS

Materials

Tyrosinase from mushroom (EC 1.14.18.1) was purchased from Sigma-Aldrich Chemie GmbH, Germany, with a specific activity of 5,370 units/mg solid. One unit represents the change in A_{280} of 0.001 per min at pH 6.5 and 25°C in 3 mL reaction mix containing L-tyrosine. The acrylamide, bisacrylamide, L-tyrosine and all the other chemicals were all reagent grade (Sigma Aldrich).

Immobilization of enzyme

The system for immobilization contained: 66.6% (v/v) of acrylamide/bisacrylamide solution [20% (w/v) acrylamide with 5.3% (w/v) bisacrylamide], 31.3% (w/v) of enzyme solution (0.275 mg protein/mL), 2.1% (v/v) of 3% (w/v) ammoniumperoxodisulfate (APS), and 60 μL of N,N,N,N'-tetramethylethylenediamine (TEMED) (modified from RAVIYAN *et al.*, 2003). The components of immobilization system were stored at 4°C until used.

The immobilized enzyme-containing gel was prepared by blending the enzyme solution into the acrylamide/bisacrylamide solution while stirring on a magnetic stirer plate with a stirring bar for 2 min at low speed. APS was added, stirred for 1 min, followed by TEMED, and stirred for 0.5 min. The mixture was allowed to polymerize between two glass plates separated with spacer for 1 hour at 4°C. In polyacryla-

mide gel polymerization, APS and TEMED are initiators which are effectors of polymerization. So, gels with 4 mm thickness and 14 cm width were formed. The gel was transparent before the polymerization reaction. During the polymerization, gel began to change its color and whole gel got the white color with the completion of the polymerization reaction. The reason of the getting white gel was the addition of the enzyme because when the gel was prepared by all other ingredients except enzyme, it was colorless. This gel obtained without enzyme was used to understand whether nonenzymatic browning occur or not during storage.

Color change

Color measurements were done using a Hunter-Lab ColorFlex (A60-1010-615 Model Colorimeter, Hunter-Lab, Reston, VA). The instrument was standardized each time with a white and black ceramic plate (L_0 = 93.01, a_0 = -1.11 and b_0 = 1.27) and L^* values of indicator were recorded which shows whiteness or brightness-darkness.

Preparation and storage of TTI

For indicator preparation, the gel was cut into 3 cm x 3 cm pieces with a razor blade knife and placed into plastic zipper bags. Then, 10 mL of L-tyrosine solutions with different concentrations (0.1, 0.5 and 1 mM) were added over them. Five replicates of indicators were stored at temperatures of 5°, 10°, 15° and 25°C in cooling incubator and color change was measured at different time intervals. The color change of enzymatic based TTI's were measured as L* by using Hunter Colorimeter. Distilled water was also added to indicator gel system in place of L-tyrosine to test whether the system gave any color change response during storage and color change was not observed. Similarly, L-tyrosine solution was added to the gel without enzyme and stored to test any color change whether could be formed. Again, no color change was observed. There were some important points to get desired, reliable enzymatic TTI production. Adjustment of enzyme and L-tyrosine concentrations to get readable color change rate and gel thickness are some of them. Substrate concentration is important for the rate of the enzyme catalyzed reaction which takes place.

TTI kinetics

Loss of shelf life in a food is usually evaluated by the measurement of a characteristic quality index, "A" The quality index for foods includes flavor, color, nutrients level, texture, and sensory quality as well as microbial load and toxin production. The change of quality index A with time t can usually be expressed as:

$$f(A) = k_A.t$$

In the case of TTI, considering lightness (L*) as the quality attribute of interest, the change of L* with time can be expressed according to LABUZA (1984) as:

$$\frac{dL^*}{dt} = -k_{brown}.L^{*n}$$

where, n is the reaction order and k_{brown} (day^{-1}) is the reaction rate constant responsible for enzymatic browning. The rate constant k_{brown} depends on temperature, T (K), according to Arrhenius' law;

$$k = k_{ref}.e^{\frac{E_A}{R}(\frac{1}{T_{ref}} - \frac{1}{T})}$$

where; k_{ref} (day^{-1}) is the rate constant at some reference temperature T_{ref} (K), Ea (J mol^{-1}) is the energy of activation of the reaction that controls quality loss and R_{gas} (8.314 J mol^{-1} K^{-1}) is the universal gas constant.

RESULTS AND DISCUSSION

Development of enzymatic TTI

Three different concentration of L-tyrosine (0.1, 0.5 and 1 mM) were used for each enzymatic TTI gel systems which were stored at 5°, 10°, 15° and 25°C. The highest L-tyrosine concentration was decided as 1 mM because substrate's maximum solubility was around 1.1 mM. The enzyme concentration was determined by the observation of color changes of enzymatic TTI's which were prepared by using different concentrations of enzyme. Optimum enzyme concentration was found as 0.275 mg protein/mL in which recordable color change rate was obtained.

Color changes of enzymatic TTI samples

When L-tyrosine solution was added, all the TTI samples showed the same color change profile at all temperatures. At the beginning of the experiment color of the TTI samples were white and then change to pale pink color and finally to the black where stable color values were measured. This color which reached at last stage was very stable and stability of color was observed for 3 months. The irreversibility of color was important for the reliability of the produced TTI. The color of TTI's turns from white to black which was due to the action of enzyme in which, tyrosinase catalyzes the conversion of L-tyrosine to quinones, probably further at the end of serial reactions to stable melanins. Rate of the reaction can well be correlated with color change of TTI gels and L* values were good indicator for this.

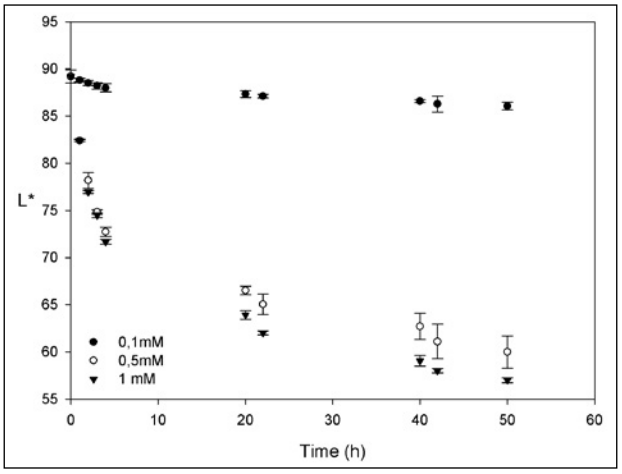


Fig. 1 - Change in L* values of TTI stored at 5°C.

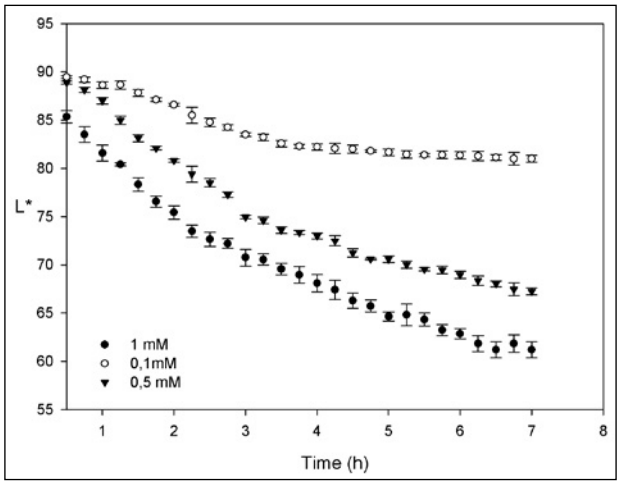


Fig. 2 - Change in L* values of TTI stored at 10°C.

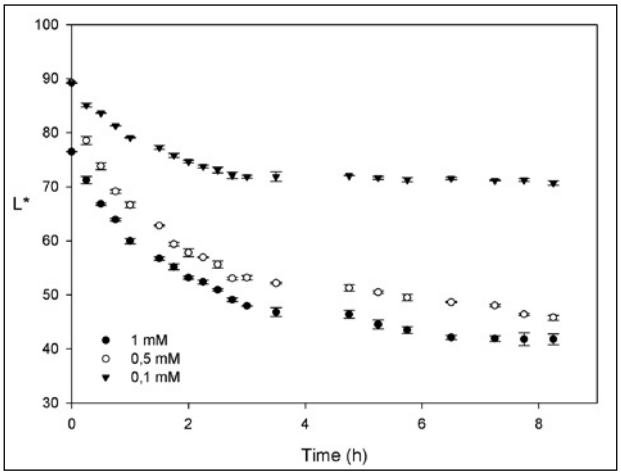


Fig. 3 - Change in L* values of TTI stored at 15°C.

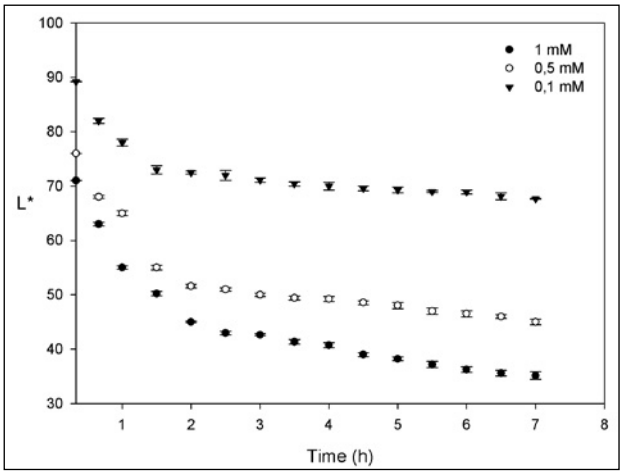


Fig. 4 - Change in L* values of TTI stored at 25°C.

Change in L* values of TTI gels stored with different L-tyrosine concentrations at temperatures of 5°, 10°, 15° and 25°C were given in Figs. 1-4. The L* values of TTI gels decreased with storage time at all temperatures and this reduction was also dependent on the L-tyrosine concentration. Increasing the substrate concentration and temperature increased the rate of enzyme catalyzed reaction and produced darker gels. After a period of time, there was no change in L* values for all TTI gels with different L-tyrosine solutions stored at different temperatures which shows the irreversible color change at the end of the reaction.

As a result we can say that increasing the substrate concentration and storage temperature increased the color changes of enzymatic TTI. The photography's of TTI's stored with different L-tyrosine concentrations at same conditions were given in Fig. 5 and it can be seen that as substrate concentration was increased, color of gel was darker.

Enzymatic TTI kinetics

Different models have been used to analyse the usefulness of a TTI to act as a quality label. TAOUKIS and LABUZA (1989a,b) developed the most frequently used approach which is based on the Arrhenius equation. It shows the correlation between the temperature dependent rate of deterioration of the quality of the product (spoilage) and the temperature dependent rate of colour change of the TTI (KREYENSCHMIDT *et al.*, 2010).

WELLS *et al.* (1987) described another approach which use a graphical interpretation, with a so called 'indicator action diagram', that simplifies mathematical models. SMOLANDER *et al.* (2004) correlated the rates of colour changes of the TTI with the microbiological counts of the food products. Additionally, they compared the TTIs' shelf life with that of the products' to evaluate the use of TTIs in monitoring food freshness (KREYENSCHMIDT *et al.*, 2010).

To be able to use a particular TTI as a quality indicator for a specific food product it is necessary that energy of activation E_a, indicating the temperature dependence of the TTI response, matches the E_a of the food product. E_a value gives a direct measure on how temperature sensitive the product is. The higher the Ea value the greater difference between a low temperature and a high. Furthermore it greatly facilitates the choice of a suitable TTI-indicator to the TTI characteristics of the product.

When L* values at different temperatures versus time at constant L-tyrosine concentration was plotted, two linear curve was obtained. It was thought that, the first linear curve was due to the enzymatic browning reaction and second curve was due to nonenzymatic browning leading to the formation of brown-black melanins. By this assumption, when L* values were plotted versus time it was seen that color change followed zero order kinetics and from the slopes of the first linear curve reaction rate constants (k) were determined for each TTI with different L-tyrosine concentrations (Table 1).

By using k values activation energies were found from Arrhenius plot. Activation energies of TTI's were determined as 123 kJ/mol (r^2=0.90), 52 kJ/mol (r^2=0.98) and 49 kJ/mol (r^2=0.99) for 0.1, 0.5 and 1 mM L-tyrosine concentration, respectively (Fig. 6). SUN *et al.* (2007) produced amylase type TTI indicator with different α-amylase concentrations (15, 20, 25 and 30%) and stored at 4°, 8°, 12°, 18°, 25° and 37°C. They determined color change process of amylase and found the activation energies of TTI ranged from 102 to 110 kJ/mol which gave somewhat narrow range of applicability. LABUZA (1982) pointed out the typical activation energy values for food quality losses ranging from 0 to 150 kcal/mol for different quality loss parameters. LABUZA and KAMMAN (1983) proposed that to ensure the error of food quality evaluation below 15%, the difference of the activation energy between the indicator and food should be less than 40 kJ/mol. In our study, activation energy values of TTI's with different concentrations of L-tyrosine were between 49 and 123 kJ/mol (11.7-29.4 kcal/mol), which gives good range of applicability in foods. By taking the maximum difference level of 40 kJ/mol of activation energies (between 2.15-38.95 kcal/mol), our TTI activation energy match with the activation energy values for food quality losses (LABUZA, 1982) as diffusion controlled (0-15 kcal/mol), enzymic (10-15 kcal/mol), hydrolysis (15 kcal/mol), lipid oxidation (10-25 kcal/mol), nutrient loss (20-30 kcal/mol), nonenzymatic browning (25-50 kcal/mol) and microbial growth (20-60 kcal/mol).

There are also commercially available time-temperature indicators which have different design, reaction type, temperature behavior and thus different range of activation energy. TAOUKIS and LABUZA (1989a) reported the activation energy ranges of LifeLine™, 3M Monitor Mark® and VITSAB®. VITSAB is based on a color change caused by a pH decrease which is the result of a controlled enzymatic hydrolysis of a lipid substrate. At activation, enzyme and substrate are mixed by mechanically breaking the barrier that separates the two compartments. Hydrolysis of the substrate causes acid release and the pH drop is translated in a color change of the pH indicator from deep

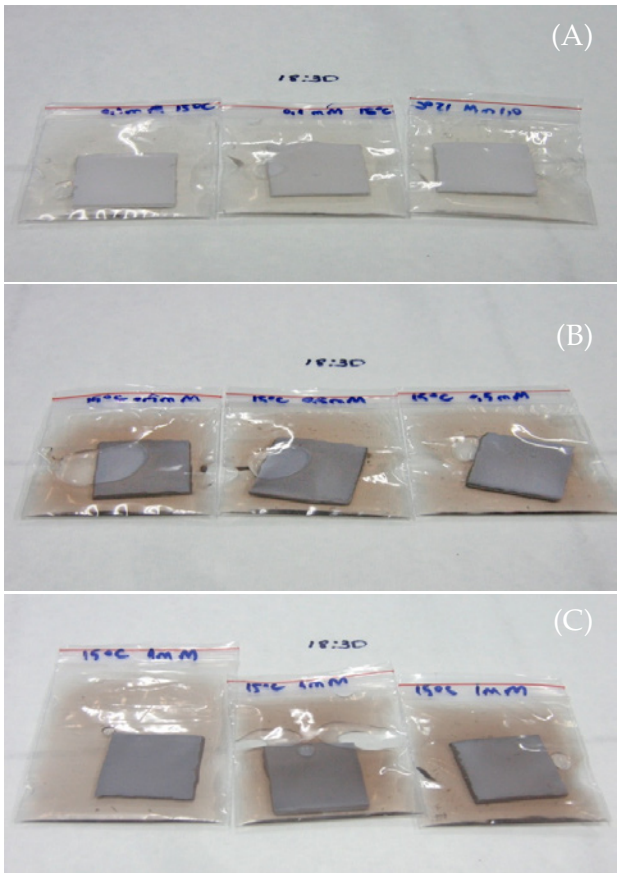


Fig. 5 - Photography of TTI stored at 15°C for 5 hours in 0.1 mM (A), 0.5 mM (B) and 1 mM (C) L-tyrosine solutions.

Table 1 - Reaction rate constants of TTI's at different storage temperatures.

Storage temperatures (°C)	Substrate concentration (mM)	k (day^{-1})
5	0.1	0.301
	0.5	4.063
	1.0	4.303
10	0.1	2.267
	0.5	5.001
	1.0	6.468
15	0.1	5.164
	0.5	8.616
	1.0	9.753
25	0.1	13.442
	0.5	17.180
	1.0	17.848

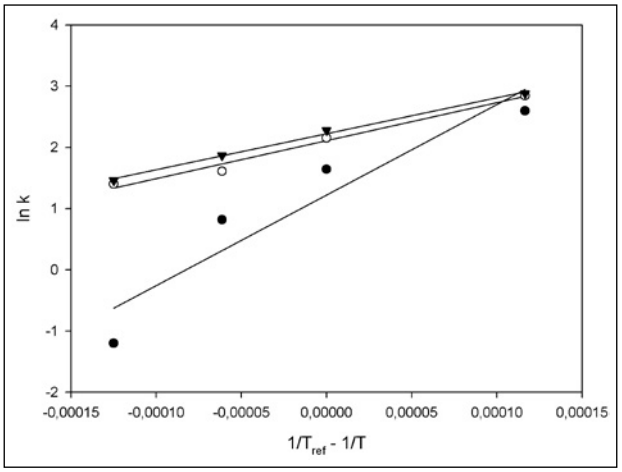


Fig. 6 - Arrhenius plots of TTI's with 0.1 mM (-○-) (r^2=0.90), 0.5 mM (-●-) (r^2=0.98) and 1 mM L-tyrosine (-▼-) (r^2=0.99).

green to bright yellow. He reported that VITSAB® has much wider range of activation energy, 12-27 kcal/mol. In VITSAB, color change is followed which is related to pH changes. In our new TTI system, change in color is visible and can be seen directly and the activation energy ranges (11.7-29.4 kcal/mol) show the possibility of using this new TTI system for various food systems.

CONCLUSION

Time temperature indicators are useful devices to monitor the cold chain and/or correlate the shelf life of food products. Because each product have its own temperature dependency and shelf life, it is necessary to develop TTIs with wider range of activation energy and also to develop specific TTI for specific food product. The basic goal of this study is to develop new enzymatic time temperature indicator. To use TTI as quality indicator of foods, activation energy and kinetics of food and TTI should match each other. Color change was used as the response parameter for TTI and activation energy was calculated from the kinetics of color change. Range of activation energy values of developed TTI well correlate with the activation energy of foods. Response time of the TTI changed with the tyrosine concentration and it can be suitable for the monitoring of refrigerated foods like poultry and meat.

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PAPER

LIPIDS AND MINERALS OF THE MOST REPRESENTED HAZELNUT VARIETIES CULTIVATED IN CROATIA

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ABSTRACT

The Croatian autochthonous hazelnut variety Istarski Duguljasti and two introduced varieties Romische Zellernuss and Hallesche Riesen were characterized with respect to their fatty acid composition as well as their squalene, tocopherols and minerals content. Oleic acid was the most abundant fatty acid ranging from 78.13% in Istarski Duguljasti to 82.98% in Romische Zellernuss, followed by linoleic acid, which showed the largest variation in the content between varieties. α-, β- and γ-tocopherol were identified in all three varieties. The level of squalene ranged from 201.2 to 243.6 mg/kg and potassium was the most abundant mineral varying between 6.81-7.77 g/kg. Obtained results highlight the nutritional characteristics of hazelnut and the influence of hazelnut variety on studied nutrients.

- Keywords: antioxidants, *Corylus avellana* L., fatty acids, minerals, squalene, tocopherols -

INTRODUCTION

Hazel (*Corylus avellana* L.) is valuable species whose fruit, the hazelnut, has extensive usage in food processing and cosmetics industry. It is consumed raw or roasted. Its ingredients are part of many industrial food products. In confectionary industry hazelnut is primarily used in chocolate production, as well as for confectionery products fillings and nougats, and for specialty products such as coated candies (OLIVER CHEN and BLUMBERG, 2008; ALASALVAR *et al.*, 2009a). Hazelnut oil can be recovered from kernel effectively by pressing, due to high fat content. It is used for flavoring confectionery, as salad oil and as a source of cooking oil (MIRALIAKBAR and SHAHIDI, 2008). Due to higher demand for diesel fuel, production of biodiesel from hazelnut oil has been studied recently (XU and HANNA, 2009; MOSER, 2012). According to FAO (FAO-STAT, 2011) data, the most significant hazelnut producers are Mediterranean countries. Turkey is the world's largest producer followed by Italy and Croatia was ranked at twelfth place. In Croatia, the majority of production is located in Istria where autochthonous variety Istarski Duguljasti originates from. In recent years, the number of intensive hazelnut plantations has increased significantly in the continental part of Croatia. One of the most represented varieties is the autochthonous variety Istarski Duguljasti.

Hazelnut varies in content of nutritional components depending on a variety, harvesting year, origin and agro-ecological conditions (KOKSAL *et al.*, 2006; SEYHAN *et al.*, 2007; BOSTAN and GUNAY, 2009; BEYHAN *et al.*, 2011; LOCATELLI *et al.*, 2011). The hazelnut contains 54.6-69.0% of fat, which is characterized by high content of unsaturated fatty acids (UFA). Their positive effects on prevention of cardiovascular diseases were confirmed (ALASALVAR *et al.*, 2009a; ROS, 2010). Several epidemiological and clinical studies suggested that frequent nut consumption is associated with reduced risk of coronary heart diseases, stroke and arteriosclerosis influencing plasma lipids profile. The main reason for this is favourable fatty acid composition (KRIS-ETHERTON *et al.*, 1999; MERCANLIGIL *et al.*, 2007; DJOUSSE *et al.*, 2010). Fat content and fatty acid composition varies depending on a variety and growing conditions (SEYHAN *et al*, 2007; ALASALVAR *et al.*, 2009b; BEYHAN *et al.*, 2011). Monounsaturated fatty acids (MUFA) constitute the majority of fatty acids present in hazelnut and oleic acid (C18:1n-9) is the predominant (73.8-84.1%). High amount of polyunsaturated fatty acids (PUFA) was found in hazelnut also. During fruit development the content of MUFA increases as the content of PUFA decreases while SFA shows no changes (SEYHAN *et al.*, 2007).

Hazelnut also contains other potentially cardio protective constituents including tocopherols, squalene, phytosterols and phytostanols (MAGUIRE *et al.*, 2004; ALASALVAR *et al.*, 2009a; ROS, 2010). Tocopherols and squalene are powerful antioxidants and radical scavengers since they bond with free radicals thus giving the stable molecules, which protect cell membranes from harmful environmental effects. Their concentration in hazelnut depends on a variety, growing conditions and fruit quality (AMARAL *et al.*, 2006; ALASALVAR *et al.*, 2009a). It is also reported that hazelnut is a good source of minerals, being particularly rich in potassium, phosphorus, calcium and magnesium. Mineral composition is largely dependent on hazelnut variety and other growing conditions (OZDEMIR and AKIHCI, 2004; KOKSAL *et al.*, 2006; ALASALVAR *et al.*, 2009b).

In the present study, the Croatian autochthonous variety Istarski Duguljasti, as well as Romische Zellernuss and Hallesche Riesen were characterized with respect to fatty acid composition, squalene, tocopherols and minerals content. These are the three major commercial hazelnut varieties cultivated in Croatia and their nutrient composition has not been described in the literature yet.

MATERIALS AND METHODS

Samples

The study was carried out on the hazelnut plantation in Orahovica (Croatia). The plantation is located in a typical fruit growing position on 140-160 AMLS (45° 33´ 30˝ N and 17° 52´ 30˝ E). All required agro-technological measures are applied regularly. The variety Istarski Duguljasti is a predominant variety in the plantation, while Romische Zellernuss and Hallesche Riesen are ancillary varieties. For each variety, 6 trees were randomly selected, the fruits were handpicked after most of them had fallen on the ground (September 2010) and were air-dried in net plastic bags for 5-7 days, protected from direct sunlight. Three representative samples of 2 kg fruits were taken for each variety for analysis. After drying, the fruits were unshelled and kept in plastic bags in the refrigerator at 5°-8°C. Analytical samples were prepared by quartering and grinding with Microtron MB 550 (Kinematica AG, Littau-Lucerne, Switzerland). The skins were not removed before grinding and grinded samples were kept in the refrigerator for maximum five days prior to analysis. The total lipids were extracted with petrol ether in a Soxhlet apparatus according to the AOAC method 948.22 (AOAC, 2000) and used for analyses of fatty acid composition, squalene and tocopherol content.

Reagents and standards

Hexane, isooctane, isopropanol and methanol gradient grade were purchased from J.T.

Backer, (Devanter, Holland). Petrol ether, p.a., nitric acid, 65%, p.a., potassium hydroxide, p.a., and sodium hydrogensulphate monohydrate, p.a., were purchased from Kemika (Zagreb, Croatia). Analytical standards were: Food Industry FAME, 37 components in methylene chloride (Restek, Bellefonte, PA, USA), squalene (Merck KG, Damstadt, Germany), α-tocopherol, β-tocopherol, γ-tocopherol and δ-tocopherol (all Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), and mineral standard solutions, 1,000 mg/L CertiPUR (Merck KG, Damstadt, Germany).

Fatty acid and squalene contents

Extracted triacylglycerols were converted to corresponding fatty acid methyl esters (FAME) by trans-esterification with methanolic solution of potassium hydroxide. Approximately 60 mg of the sample was weighed into test tube with glass stopper and dissolved by 4 mL of isooctane. After that, 200 μL of potassium hydroxide solution in methanol (2 mol/L) was added and the reaction was carried out at room temperature with vigorous shaking (2 x 30 s). The solution was neutralized by addition of 1 g of sodium hydrogensulphate monohydrate and transferred into 2 mL vial. Fatty acid composition and squalene content were determined on CP-3800 gas chromatograph (Varian, Palo Alto, CA, USA) equipped with the flame ionization detector, capillary column DB-23 60 m x 0.25 mm, 0.25 μm film thickness (Agilent Technologies, Palo Alto, CA, USA) and split/splitless injector. The injector temperature was 250°C and the injections were performed with split ratio 1:30. Helium was used as carrier gas at a flow rate of 1.5 mL/min and the temperature program of the column oven was: 60°C (1 min) rising to 220°C at the rate of 7°C/min. FAMEs and squalene were identified by comparison with the retention times of standards listed in Reagents and standards section and calculated by normalization procedure. A detailed description of the method and its suitability was given previously (PETROVIĆ *et al.*, 2010).

Tocopherol contents

The tocopherol content of hazelnut oil samples, extracted in Soxhlet apparatus, was determined according to European Norm 12822 (European Norm, 2000). 2 g of sample was accurately weighed into 25 mL volumetric flask, dissolved in n-hexane on ultrasonic bath and the flask was filled to the volume with n-hexane. The obtained sample solutions were analyzed by normal phase HPLC on SpectraSystem 2000 (Thermo, Bellefonte, PA, USA). The chromatographic separation was achieved with Supelcosil LC-Si column, 250 mm x 4.6 mm, 5 μm (Supelco, Bellefonte, PA, USA) at room temperature. The mobile phase was n-hexane:isopropanol 99:1 at 1 mL/min and the injection volume was 20 μL. Detection was performed on fluorescence detector with excitation wavelength of 296 nm and the emission wavelength of 330 nm. Tocopherols were identified by comparing retention times of obtained peaks with retention times of peaks in chromatogram obtained by injection of tocopherol standard solution. The results were calculated by external standard method.

Mineral contents

Prior to analysis of mineral content, samples were ashed by microwave digestion. Approximately 500 mg of homogenized sample was transferred to polytetrafluoroethylene vessel followed by addition of 8 mL of 65% nitric acid. Digestion was performed in Ethos 1600 Microwave Digester (Milestone Corp., Sorisole, Italia) using the following program: microwave power was programmed in three steps (250, 400 and 600 W) at 5 min intervals. Digests were diluted to 20 mL with deionized water. Calcium and magnesium were analyzed according to the AOAC method 975.03 (AOAC, 2000) on Solaar Thermo Scientific Spectrometer GF 95 (Thermo, Bellefonte, PA, USA) in an air-acetylene flame. Potassium was analyzed according to the AOAC method 980.83 (AOAC, 2000) on PFP7 Jenwav (Bibby Scientific Limited, Stone, Staffordshire, UK) by flame photometric method. Phosphorus was analyzed according to the AOAC method 986.24 (AOAC, 2000) on PU 8600 series UV/VIS (Pye UNICAM, Cambridge, UK).

Statistical analysis

The results were expressed as mean ± standard deviation (SD) of six replicates for each sample. The statistical significance of obtained results was tested by t-test (two samples with equal variance, two-tailed distribution) using SAS statistical software, version 8.2 (SAS Institute Inc., Cary, NC, USA). A p-value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Fatty acids composition

The pomological traits and proximate chemical composition of the most represented hazelnut varieties cultivated in Croatia were reported in the previous study (VUJEVIĆ *et al.*, 2010) where the data on fat content were also given. Results of the research indicated that fat was predominant component of the hazelnut (61.60-67.59%). In this research fatty acid composition of hazelnut oil obtained from three hazelnut varieties was studied (Table 1).

Table 1 - Fatty acid composition (%) of oil extracted from hazelnut samples.

Fatty acids (%)	Istarski duguljasti	Romische Zellernuss	Hallesche Riesen
C12:0	Tr	Tr	Tr
C14:0	0.04±0.01[a]	0.04±0.01[a]	0.03±0.01[b]
C15:0	Tr	Tr	Tr
C16:0	5.87±0,32[a]	5.44±0.25[b]	5.48±0.18[b]
C16:1n-9	0.04±0.01[a]	0.03±0.01[a]	.03±0.01[a]
C16:1n-7	0.23±0.01[a]	0.16±0.02[b]	0.17±0.01[b]
C170	0.05±0.00[a]	0.05±0.00[a]	0.05±0.00[a]
C17:1n-10	0.09±0.01[a]	0.07±0.01[b]	0.07±0.01[b]
C18:0	2.42±0.21c	3.49±0.30[a]	2.84±0.14[b]
C18:1n-9	78.13±1.73c	82.98±0.62[a]	80.92±0.67[b]
C18:1n-7	1.13±0.03[a]	0.75±0.02c	0.90±0.04[b]
C18:2n-6	11.59±2.01[a]	6.59±0.30c	9.10±0.59[b]
C18:3n-3	0.10±0.01[a]	0.10±0.01[a]	0.11±0.01[a]
C20:0	0.12±0.01[b]	0.15±0.03[a]	0.13±0.01[ab]
C20:1n-9	0.16±0.01[a]	0.13±0.01c	0.15±0.01[b]
C22:0	0.02±0.01[a]	0.02±0.01[a]	0.03±0.01[a]
Total SFA	8.52±0.49[b]	9.19±0.38[a]	8.55±0.16[b]
Total MUFA	79.77±1.74c	84.12±0.60[a]	82.23±0.68[b]
Total PUFA	11.69±2.03[a]	6.69±0.31c	9.21±0.59[b]
C18:1n-9/C18:2n-6	6.95±1.47c	12.62±0.68[a]	8.93±0.67[b]
MUFA/PUFA	7.03±1.49c	12.60±0.68[a]	8.97±0.65[b]
MUFA/SFA	9.39±0.47[ab]	9.17±0.44[b]	9.62±0.24[a]

Fatty acid contents are expressed as means ± SD (n=6). Means ± SD followed by the same letter are not significantly different (p>0.05).
Tr: trace, detected values were less than quantification limit of the method (LOQ=0.01%).
SFA, saturated fatty acids, MUFA, monounsatured fatty acids, PUFA, polyunsatured fatty acids.

MUFA were predominant among sixteen fatty acids identified in the samples. Oleic acid was the most abundant fatty acid ranging from 78.13% in Istarski Duguljasti to 82.98% in Romische Zellernuss. The second most abounded fatty acid was linoleic acid, which showed the largest variation in the content of varieties. Fig. 1 shows the content of oleic acid compared to linoleic acid. It is evident that the content of these two fatty acids was inversely dependent and the obtained correlation coefficient (r=-0.9855, p>0.05) was significant. SEYHAN *et al.* (2007) that the ratio between oleic and linoleic acid was inversely related during fruit development. Grouping of the data is also noticeable mainly due to large differences between varieties in the linoleic acid content. This has substantially affected the ratio of oleic and linoleic acid content as well as MUFA/PUFA ratio. MUFA/SFA ratio is less dependent on varieties and the obtained correlation coefficient (r=0.5390, p<0.05) was not significant. Palmitic acid, stearic acid and vaccenic acid (isomer of oleic acid, C18:1n-7) were also present ranging from 0.75 to 5.87%, while the amount of all other fatty acids was less than 0.3%.

Presented data clearly show that fatty acid content differs among hazelnut varieties. This is in agreement with other reports, and the obtained values were within ranges reported in the literature (OZDEMIR *et al.*, 2004; KOKSAL *et al.*, 2006; OLIVEIRA *et al.*, 2008; LOCATELLI *et al.*, 2011). PARCERISA *et al.* (1998) confirmed the important influence of varieties on fatty acid composition and confirmed grouping of hazelnut varieties according to their origin by discriminant analysis of individual fatty acid content as a variable. The study of CREWS *et al.* (2005) is the only study that deals with the Croatian hazelnuts. Compared to our results, the main differences are in the content of palmitic and linolenic acid. Besides the possible difference that could arise from not identified hazelnut variety in the report, a possible reason for this could be the difference in harvesting season and growing conditions (ALASALVAR *et al.*, 2009a; BEYHAN *et al.*, 2011).

Antioxidants

Tocopherols and squalene are the main lipophilic compounds with antioxidant activity in hazelnut. Antioxidant composition of studied hazelnut varieties was presented in Table 2. The results indicated that the α-tocopherol had the highest content among tocopherol isomers ranging from 268.0 mg/kg in Istarski Duguljasti to 329.8 mg/kg in Hallesche Riesen. All samples contained β- and γ-tocopherol but in significantly smaller amounts. δ-tocopherol found in other studies was not detected in our samples and the limit of detection of the method was 1.0 mg/kg of oil. Total tocopherol ranged between 277.7 and 335.6 mg/kg of oil.

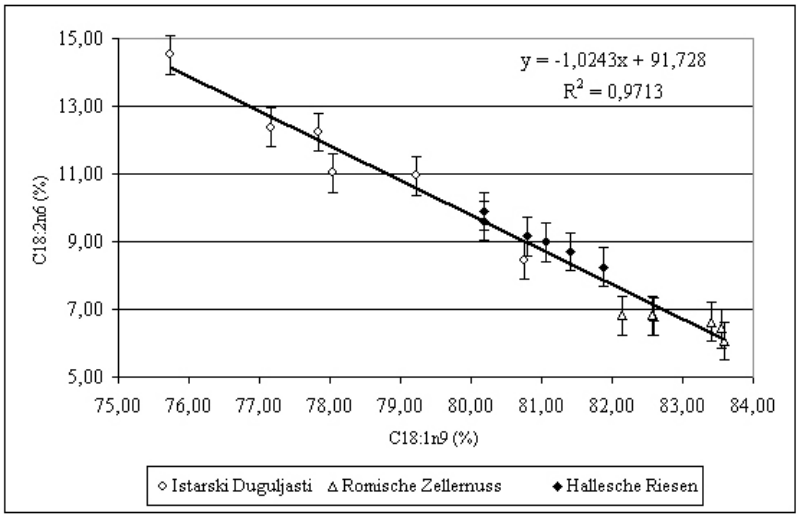


Fig. 1 - Correlation between oleic acid content and linoleic acid content.

The results of our study were in agreement with the range of values obtained by other authors, although the results are dependent on variety and growing conditions (CREWS *et al.*, 2005; ALASALVAR *et al.*, 2006; AMARAL *et al.*, 2006). The average content of total tocopherols ranged between 298 to 513 mg/kg in hazelnut oil from Turkey and α-tocopherol was predominant. Our results are in the closest agreement with the research of hazelnut grown in Portugal (ACKURT *et al.*, 1999; AMARAL *et al.*, 2006) due to the similar ratio between α-tocopherol content and the content of β- or γ-tocopherol. They also reported δ-tocopherol content in the range of 0.7-1.5 mg/kg, which is at the detection limit of our method. In the study of CREWS *et al.* (2005) the hazelnut sample from Unije, Croatia was included also. Total tocopherols level was 405 mg/kg, γ-tocopherol level was 65 mg/kg, while β-tocopherol was not found. In comparison with our results, total tocopherols were significantly higher. The percentage of γ-tocopherol in total amount of tocopherols contained in the sample was higher for approximately one order of magnitude: 16.0% in comparison to the range 1.0-2.8% that we obtained. That difference could be explained by variation in harvest year, growing conditions and variety of the sample from Unije which was not mentioned. ACKURT *et al.* (1999) reported the average value of total tocopherols in hazelnut of 353 mg/kg, with no significant differences observed per varieties. In our research, however, differences among varieties were observed.

Apart from high tocopherol content, high content of squalene was determined in hazelnut as well. Although hazelnut is a rich source of squalene, such data is limited. According to BENITEZ-SANCHEZ *et al.* (2003) research, squalene content in hazelnut oil ranges from 146-245 mg/kg. This makes them a significant source of squalene, even though the amount is 20 times less than in olive oil. MAGUIRE *et al.* (2004) tested chemical composition of frequently consumed nuts and reported 186.4 mg/kg of squalene in hazelnut. The results of this study, presented in Table 2, were in agreement with results reported in the literature. The highest level was found in Romische Zellernuss and the results for Istarski Duguljasti and Hallesche Riesen were not significantly different.

Table 2 - Tocopherol and squalene composition (mg/kg) of oils extracted from hazelnut samples.

Tocopherols (mg/kg)	Istarski duguljasti	Romische Zellernuss	Hallesche Riesen
α-Tocopherol	268.0±23.7c	290.5±23.8^{b}	329.8±14.7^{a}
β-Tocopherol	1.9±0.7^{a}	2.5±0.4^{a}	2.4±0.6^{a}
γ-Tocopherol	7.8±1.4^{a}	5.6±0.8^{b}	3.3±0.4c
Total Tocopherols	277.7±25.5^{b}	298.6±23.5^{b}	335.6±14.1^{a}
Squalene	201.2±39.4^{b}	243.3±28.6^{a}	208.6±40.2^{b}

Tocopherol contents are expressed as means ± SD (n=6). Means ± SD followed by the same letter are not significantly different (p>0.05).

Table 3 - Mineral content of hazelnut samples.

Minerals (g/kg)	Istarski duguljasti	Romische Zellernuss	Hallesche Riesen
Calcium	2.51±0.26[a]	2.43±0.27[ab]	2.20±0.20[b]
Magnesium	1.88±0.13[b]	2.02±0.18[ab]	2.10±0.23[a]
Phosphorus	2.91±0.18[b]	3.25±0.15[a]	2.96±0.14[b]
Potassium	6.81±0.25[b]	7.77±0.28[a]	7.69±0.08[a]

Data are expressed as means ± SD (n=6). Means ± SD followed by the same letter are not significantly different (p>0.05).

Mineral composition

Mineral content of hazelnut varieties was significantly dependent on the variety (Table 3). Potassium was the most abundant mineral ranging from 6.81 g/kg in Istarski Duguljasti to 7.77 g/kg in Romische Zellernuss which also had the highest content of phosphorus. Calcium content was the highest in Istarski Duguljasti which had the lowest content of magnesium. Contrary to this, Hallesche Riesen had the lowest mean value for calcium content and the highest mean value for magnesium content. Correlation between calcium and magnesium content was tested and the obtained correlation coefficient (r=0.2641, p<0.05) was not significant.

The influence of varieties and production parameters on mineral content was investigated in several studies (ACKURT *et al.*, 1999; OZDEMIR *et al.* 2004; KOKSAL *et al.*, 2006; SEYHAN *et al.*, 2007; ALASALVAR *et al.*, 2009b). SEYHAN *et al.* (2007) reported that content of all studied minerals decreased significantly during fruit development and that the mineral content in the harvest stage was within the range obtained by other authors. Their values were quite different from ours. The content of all minerals except phosphorus was lower than our values. Our results were in accordance with the study of OZDEMIR *et al.* (2004) although they did not get a significant difference among studied varieties for calcium and phosphorus content. A close agreement was also obtained with the results of ALASALVAR *et al.* (2009b) and with the results of KOKSAL *et al.* (2006) who reported the greatest differences in mineral content among samples in their study on 17 different hazelnut varieties.

CONCLUSION

The results of this study confirm that hazelnut is an abundant source of nutrients, and is rich in unsaturated fatty acids, antioxidants and minerals. Statistical analysis of the obtained data pointed out that there was significant difference in chemical composition among varieties grown in the same field. This confirms that differences in varieties affect the chemical composition, since all three varieties were grown under the same conditions. Oleic acid was predominant fatty acid in hazelnut. Its content and the content of linoleic acid were inversely dependent. Antioxidant composition of studied hazelnut varieties was in accordance with the range of values reported in other studies, which could be concluded for mineral composition as well. This paper contributes to updating databases on hazelnut chemical composition.

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PAPER

PROBIOTIC CHARACTERISTICS OF NATURAL LACTOBACILLI ISOLATED FROM TRADITIONAL KARGI TULUM CHEESE

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ABSTRACT

In this study, the probiotic characteristics of *Lactobacillus* spp. (75 strains) isolated from traditional Kargi tulum cheese were determined. First, their acid (pH 1-3), bile (0.3-1%) and simulated gastric juice (pepsin 0.3% w/v, NaCl 0.5% w/v, pH=2) tolerances were determined, and 11 strains (7 *L. paracasei*, 3 *L. plantarum*, and 1 *L. brevis*) that survived in these conditions were selected for further examination. API 50CH and API-ZYM test results showed that these 11 strains could ferment numerous sugars and had a wide range of enzyme activities, including β-galactosidase. In addition, bacteriocins obtained from *L. plantarum* KT45, KT68 and KT110 inhibited *Escherichia coli*, *E. coli* O157:H7, *B. cereus*, *Salmonella* sp. and MRSA. Disk diffusion test results showed that *L. plantarum* KT45, KT68 and KT110 and *L. paracasei* KT39 were sensitive to streptomycin, erythromycin, gentamicin, chloramphenicol, tetracycline, rifampicin, amoxicillin, vancomycin and penicillin and were resistant to nalidixic acid. These strains were also relatively hydrophobic (48.1-58.6%), and they produced EPS and H_2O_2 (0.5-2.9 μL/mL). It was concluded that the most promising probiotic strains were *L. plantarum* KT45, KT68 and KT110 and *L. paracasei* KT39.

- Keywords: *Lactobacillus*, probiotics, bacteriocin, tulum cheese -

INTRODUCTION

Probiotics are living microorganisms that, when consumed in adequate amounts, have a positive influence on the health of a host (FAO/WHO, 2002). Species belonging to the genera *Bifidobacterium* and *Lactobacillus* are commonly used probiotics (HARISH and VARGHESE, 2006). Probiotics should have the following characteristics: resistant to acid or bile, viable in the gastrointestinal system, adherent to mucosal surfaces, antimicrobial activity against pathogenic bacteria, good biotechnological properties (remain viable in food, feed and dietary supplements, resistant to phage, strain stability, culturable on a large scale, resistant to oxygen, no negative effects on the flavor of the product) and do not harbor any transferable antibiotic resistance genes (OUWEHAND *et al.*, 1999; OUWEHAND *et al.*, 2001; VON WRIGHT, 2005). In addition, the probiotic bacterium should have been isolated from the same host in which it is intended to be used, and it should have demonstrable positive effects for the host and not be pathogenic (HARISH and VARGHESE, 2006). However, there is no requirement that a potential probiotic species should have all these mentioned properties (OUWEHAND *et al.*, 1999).

There are number of studies that address probiotics and their benefits for health. The proposed beneficial health effects of probiotics include managing lactose intolerance, improving the immune system, preventing colon cancer, reducing cholesterol and triacylglycerol plasma concentrations, lowering blood pressure, reducing inflammation, reducing allergic symptoms, yielding beneficial effects on mineral metabolism (in particular, enhancing bone density and bone stability), reducing *Helicobacter pylori* infection, inhibiting pathogenic microorganisms, preventing osteoporosis, preventing urogenital infections and treating hepatic encephalopathy (HE) (FAO/WHO, 2002; PARVEZ *et al.*, 2006).

One of the notable properties of probiotic microorganisms is that they produce compounds with antagonistic effects against pathogens and saprophytes. Among these compounds are most of the metabolites produced by lactic acid bacteria (LAB), including organic acids (lactic acid, acetic acid, propionic acid), carbon dioxide, hydrogen peroxide, diacetyl, low molecular weight antimicrobial compounds, and bacteriocins, which have antimicrobial activity (OUWEHAND and VESTERLUND, 2004; ŠUŠKOVIC *et al.*, 2010).

Bacteriocins are ribosomally synthesized peptide or protein metabolites that are produced by bacteria and generally have antimicrobial activity against closely related species (NES *et al.*, 2007). The genes that encode bacteriocin biosynthesis pathways may be located on the chromosome, on plasmids or on transposons (CHEN and HOOVER, 2003). Bacteriocins differ from most therapeutic antibiotics in being proteinaceous compounds that are rapidly digested by proteases in the human digestive tract (SAAVEDRA *et al.*, 2004). In contrast to the antibiotics currently being used, bacteriocins are often considered more "natural" because they are thought to have been present in many foods eaten since ancient times (CLEVELAND *et al.*, 2001). There has been much interest in bacteriocins derived from Gram-positive bacteria because they are frequently found in many commercially useful LAB (e.g., lactococci, lactobacilli, and pediococci), and they are GRAS for human consumption. Moreover, they are non-toxic to eukaryotic cells and have a much broader inhibitory spectrum than bacteriocins derived from Gram-negative bacteria (NES *et al.*, 2007). The only bacteriocin currently approved by the FAO and WHO for use in certain foods is nisin, produced by *Lactococcus lactis* subsp. *lactis* (MILLS *et al.*, 2011; NEL *et al.*, 2004). In 1988, the Food and Drug Administration (FDA) included nisin on the list of GRAS metabolites (FDA, 1988).

In Turkey, numerous traditional fermented dairy products are produced without using starter cultures. Kargi Tulum cheese is an artisanal cheese manufactured in the high plateau of Çorum, in the Middle Anatolian region of Turkey. This cheese is manufactured in the summer months and marketed in public bazaars during the autumn (DINKCI *et al.*, 2007). It is produced by small-scale dairies using raw whole milk from sheep, goats, cows and buffalo or a mixture of milk from these animals. Curds are obtained with the natural microflora of the milk. Then, the curds are compressed into goat-skin bags to release trapped air. Goat-skin bags are traditionally used to package tulum cheese. Tulum cheese prepared in this way is then matured for at least 3 months (at 4° to 6°C) in the uplands of Kargi.

Within the scope of our project, natural LAB of Kargi Tulum cheese were previously identified on a species level using species-specific PCR primers, and their starter culture characteristics were reported (KUNDUHOGLU *et al.*, 2012). The objective of the current study was to evaluate some *in vitro* probiotic characteristics of *Lactobacillus* spp. isolated from tulum cheese. Therefore, tolerance to acid, bile and simulated gastric juice (GJ), hydrophobicity, ability to produce H_2O_2, bacteriocins and exopolysaccharides (EPS), ability to hydrolyze casein, antibiotic susceptibilities, enzymatic capacity and carbohydrate fermentation profiles of the *Lactobacillus* spp. (75 strains) were determined. Consequently, the findings obtained from this study may help to select strains with probiotic potential to act as starter cultures for manufacturing functionally probiotic Kargi tulum cheese.

MATERIALS AND METHODS

Isolation and maintenance of *Lactobacillus* spp.

The *Lactobacillus* spp. used in this study were isolated and identified at the species level with species-specific PCR primers as part of our previous study (BAP-Project number: 2007-19027). Stock cultures of *Lactobacillus* strains were maintained in MRS broth containing 15% (v/v) glycerol (Merck, Darmstadt, Germany) at -20°C.

Survival at different pH levels

Depending on the type and quantity of food, digestion can last up to 2-4 hours in the stomach (pH 1.0-2.5), 5-6 hours in the small intestine (pH=6.6 ±0.5), and 12-24 hours in the large intestine (pH 7.0±0.7) (EVANS *et al.*, 1988; MORELLI, 2000). In other words, the pH in the GI environment ranges from pH 1 to pH 8 during the digestive process. Therefore, our *Lactobacillus* strains need to survive in the acidic environment of the stomach. To simulate the pH conditions of the stomach, the pH of the MRS broth was adjusted to 1.0, 2.0 and 3.0 with 1N HCl. These broths were inoculated with each strain at a final concentration of 10^8 colony forming units per milliliter (CFU/mL) and incubated at 37°C for 4 h. After the incubation period, a 1 mL sample was removed from each culture tube and serially (1/10) diluted in phosphate buffer (pH 6.5). The dilutions were plated on MRS agar (Merck, Darmstadt, Germany). The plates were incubated at 37°C for 48 h, and then the colonies were counted. *Lactobacillus* strains cultivated in MRS broth at the standard pH (pH 5.7) served as a control. The survival (%) was calculated by comparing the final cell numbers in MRS broth at different pH levels (pH 1.0, 2.0 and 3.0) to those in MRS broth at the standard pH level.

Each *Lactobacillus* strain that survived in the acidic MRS broth (pH 1.0-3.0) was inoculated into MRS broth at pH 6.0 and 8.0. Culture tubes were incubated at 37°C for 24 h. After the incubation period, a 1 mL sample was removed from each tube and serially diluted in phosphate buffer (pH 6.5). Colony counts were determined as described above.

Survival in bile

Lactobacillus strains were cultured in 10 mL MRS broth at 37°C for 24 h. Then, the cultures were centrifuged at 6,000 rpm for 10 min and resuspended in MRS broth. Each strain was inoculated (10^8 CFU/mL) into MRS broth supplemented with 0.3, 0.5 or 1.0% (w/v) bile (Merck, Darmstadt, Germany) and incubated for 6 h at 37°C. Following the incubation, a 0.1 mL sample was removed from each culture tube, and decimal dilutions were prepared. The dilutions were plated on MRS agar and incubated at 37°C for 48 h. The percentage survival in bile was determined as described above.

Survival in simulated GJ

Simulated GJ was prepared by suspending pepsin (0.3% w/v) in 100 mL of 0.5% NaCl and adjusting the pH of the medium to 2.0 with concentrated HCl. The preparation was sterilized with a filter (0.22 μm pore size, Millipore, Bedford, MA, USA). To prepare inocula, *Lactobacillus* strains were cultured in 10 mL MRS broth at 37°C for 24 h. Cultures were then centrifuged at 6,000 rpm for 10 min and washed twice in sterile saline (NaCl 0.85%, pH 6.5). Pellets were resuspended in saline and inoculated (10^8 CFU/mL) into 10 mL of GJ. After incubation at 37°C for 6 h, a 1 mL sample was removed from each culture tube and serially diluted in phosphate buffer. The dilutions were plated on MRS agar and incubated at 37°C for 48 h. Then, colony counts and percentage survival in GJ were calculated as described above.

EPS production

To determine the EPS production capability of the *Lactobacillus* strains, MRS broth was prepared using sucrose (20 g/L) instead of glucose. Sucrose-MRS agar plates were inoculated with each *Lactobacillus* strain and incubated at 37°C for 24 to 72 h. Following the incubation period, strains that produced slimy, mucoid colonies on the agar medium were regarded as EPS producers.

H_2O_2 production

A standard curve of H_2O_2 was prepared. A stock solution of H_2O_2 (0.01 μL/mL) was prepared using a 30% H_2O_2 (Merck, Darmstadt, Germany) solution. Then, two-fold dilutions were made from this stock solution, and their absorbance was read at a wavelength of 350 nm. A standard curve was obtained by plotting the absorbance of samples containing known concentrations of the H_2O_2. Each *Lactobacillus* strain was cultured in MRS broth (37°C for 24 h). Then, 5 mL of sterile, distilled water was added to each culture tube. The tubes were centrifuged for 10 min at 6,000 rpm, and the supernatants were filtered through cellulose acetate membrane filters (0.45 μm pore size, Millipore). Then, 0.5 mL of a citric acid solution, 0.5 mL of an ammonium molybdate solution and 0.5 mL of a potassium iodide solution was added to 4 mL of each filtrate. The optical density of these mixtures was determined spectrophotometrically at a wavelength of 350 nm. The concentration of H_2O_2 present in the culture supernatant was calculated as μL H_2O_2/mL supernatant using the standard curve.

Ability to hydrolyze casein

Skim milk agar (10% w/v skim milk; 2% w/v agar) was used to determine the ability of our *Lactobacillus* strains to hydrolyze casein (PAILIN *et al.*, 2001). Briefly, *Lactobacillus* strains incubated in MRS broth for 24 h at 37°C were centrifuged at 6,000 rpm for 10 min, washed with peptone water (0.1%), and resuspended in 1 mL of peptone water. Then, 2 μL of these concentrated culture solutions were pipetted onto skim milk agar plates and incubated for 24-48 h at 37°C. Clear halos around the colonies indicated the hydrolysis of casein.

Cell surface hydrophobicity

The *in vitro* cell surface hydrophobicity was measured according to the method described by TAMANG *et al.* (2007). Briefly, *Lactobacillus* strains were grown in MRS broth at 37°C for 18-24 h. Bacterial cultures were harvested by centrifugation at 6,000 rpm for 10 min, washed twice and resuspended in 50 mM K_2HPO_4 buffer (pH 6.5) to an optical density (OD_{560}) of 0.8-1.0 (A0) as measured spectrophotometrically. Then, 0.6 mL of n-hexadecane was added to 3 mL of each bacterial suspension. The mixture was vortexed for 2 min. The tubes were allowed to stand at 37°C for 30 min to separate the two phases. The aqueous phase was carefully removed, and the OD_{580} of the aqueous phase (A) was measured. Hydrophobicity was calculated from three replicates as the percent decrease in the optical density of the initial aqueous bacterial suspension due to cells partitioning into the hydrocarbon layer. The percent cell surface hydrophobicity (H%) of an isolate partitioning into hexadecane was calculated using the equation:

$$H\% = [(A_0 - A)/A_0) \times 100]$$

Ability to produce bacteriocins

The antimicrobial activities of bacteriocins produced by *Lactobacillus* strains against indicator test bacteria, which consisted of the pathogens and LAB isolates listed in Table 4, were tested using the agar well diffusion method. *Lactobacillus* strains were cultured in 5 mL of MRS broth at 37°C for 24 h. Cultures were centrifuged at 6,000 rpm for 10 min to obtain the cell free supernatants (CFSs). The pH of the CFS was adjusted to 6.5 using 5 N NaOH to eliminate the antimicrobial effect of organic acids, and catalase was added at a final concentration of 1 mg/mL to eliminate the inhibitory activity of H_2O_2. The catalase-treated CFSs were filtered through membrane filters (0.45 μm pore size), incubated for 2 h at 37°C, heated at 70°C for 20 min and stored at 4°C. The remaining activities of the CFSs were determined against the indicator test bacteria.

Indicator test bacteria were grown in MRS (for LAB strains) or brain heart infusion broth (BHIB, for pathogenic strains) and incubated for 24-48 h at the optimum temperature of the indicator bacteria used. Prepoured MRS agar or BHI agar plates were overlaid with 7 mL of MRS or BHI soft agar (0.8% agar) containing 0.1 mL of the indicator cultures (10^8 CFU/mL). Wells (10 mm diameter) were cut into the agar plate using a sterile borer (6 mm diameter), and 100 μL of CFS was placed in each well and stored at 4°C for 1 h. The plates were subsequently incubated for 24-48 h at the optimum temperature of the indicator bacteria used. Finally, the diameter of the inhibition zones on the plates was measured in millimeters.

Partial characterization of bacteriocins

All the enzymes used in this study were purchased from Sigma. CFSs were treated with the following enzymes at a final concentration of 1 mg/mL: lipase, amylase, α-chymotrypsin, pepsin, trypsin, and proteinase K. The CFS samples (1 mL) were treated with 1 mL of each enzyme for 2 h at 37°C. These CFS + enzyme mixtures were heated at 70°C for 20 min. After enzymatic treatments, the remaining activities of the CFSs were determined against the indicator test bacteria.

Antibiotic susceptibility

The disc diffusion method (NCCLS, 2002) was used to determine the sensitivity of the *Lactobacillus* strains to therapeutic antibiotics. Ten different antibiotics were used in this study including, streptomycin, erythromycin, gentamicin, chloramphenicol, tetracycline, rifampicin, amoxicillin, penicillin G, vancomycin and nalidixic acid. MRS agar plates were swabbed with culture suspensions (10^8 CFU/mL) of *Lactobacillus* strains using a sterile cotton swab. Antibiotic discs were placed onto the surface of the inoculated agar plate. The diameter of the inhibition zone around the disk was measured after the plate was incubated for 24 h at 37°C. Resistance was defined as the absence of a zone of inhibition around the disk.

Carbohydrate fermentation and enzyme activity profiles of *Lactobacillus* spp.

Carbohydrate fermentation and enzyme activity profiles of isolates were determined using API 50CH and API-ZYM (BioMérieux, Inc., Marcy l'Etoile, France) kits according to the manufacturer's instructions.

Statistical analysis

Experiments were replicated at least three times. One-Way Analysis of Variance (ANOVA)

was used to calculate standard deviations of test results using SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

The *Lactobacillus* spp. used in this study were isolated from Tulum cheese and identified on a species level using species-specific primers. The starter culture potential of these isolates was reported in a previous study. In this study, the probiotic potential of a total of 75 *Lactobacillus* isolates was evaluated.

The selection of probiotic isolates has generally been based on their *in vitro* tolerance of several physiologically relevant stresses, including acidic pH and bile salts. Therefore, our *Lactobacillus* strains needed to survive in the acidic conditions of the stomach and in the low bile salt concentrations in the small intestine. Of the 75 isolates, only 17 were resistant to acid conditions (pH 2 to pH 3); however, only *L. plantarum* KT45, KT68 and KT110 and *L. paracasei* KT39 survived at pH 1 (Table 1). To simulate the pH of the small and large intestine, the growth of the 17 *Lactobacillus* strains that were resistant to acid was checked at pH 6 and 8, and they all showed good growth. In addition, their tolerance to bile and GJ was examined. Bile tolerance test results showed that all of the isolates were resistant to 0.3-1% bile salts. However, the percentage of survivors decreased as the bile concentration increased. As a result, 11 *Lactobacillus* strains that were able to survive in acid (pH 2-3), bile and simulated GJ were selected for use in subsequent experiments.

It has been reported that some LAB isolates produce H_2O_2 in the presence of oxygen, which has an inhibitory effect on numerous microorganisms (ŠUŠKOVIC *et al.*, 2010). Therefore, H_2O_2 producing LAB isolates could be beneficial in preventing the growth of food-borne pathogens and act as probiotics in human and animal health (KANG *et al.*, 2005). In our study, the *Lactobacillus* strains produced varying levels (0.5-2.9 μL/mL) of H_2O_2 (Table 2).

Casein constitutes more than 80% of the total milk protein, and the remaining 20% is predominately whey protein (VARNAM and SUTHERLAND, 1994). Among the many health-related properties of fermented foods, casein hydrolysis mediated by lactic acid bacteria has been described as having an effect on blood pressure. Peptides derived from casein have also been shown to have antimicrobial activity, primarily against Gram-negative bacteria (HAUSER *et al.*, 2007). In our study, casein hydrolysis test results showed that our *Lactobacillus* strains were able to hydrolyze casein (Table 2).

Cell surface hydrophobicity and EPS production are the primary factors that affect the attachment of microbial cells to different surfaces (DONLAN, 2002). The EPS may both strengthen adhesion to the substratum and lead to cell-cell attachment. The composition and quantity of EPS often vary based on the type of microorganism (PATEL *et al.*, 2010). In addition, it has been reported that the EPS from probiotic bacteria adsorbs heavy metal ions (BHAKTA *et al.*, 2012) and mycotoxins (EL NEZAMI *et al.*, 2002;

Table 1 - The survival of *Lactobacillus* strains (n=17) under different pH conditions and their tolerance to GJ and bile.

Isolate No.	Species names	Survival (%) of isolates under different conditions								
		4 h			24 h		6 h			6 h
		pH 1	pH 2	pH 3	pH 6	pH 8	0.3%bile	0.5%bile	1%bile	GJ
KT10	*L. plantarum*	–	+	+++	+++	++	++	++	+	–
KT15	*L. paracasei*	–	+	+++	+++	++	++	++	+	–
KT36	*L. paracasei*	–	+	+++	+++	++	+++	+++	++	++
KT37	*L. brevis*	–	+	+++	+++	++	+++	+++	++	++
KT38	*L. paracasei*	–	+++	+++	+++	++	+++	+++	+++	+++
KT39	*L. paracasei*	+	++	+++	+++	++	+++	+++	++	++
KT42	*L. plantarum*	–	+	+++	+++	++	+++	+++	+	–
KT43	*L. paracasei*	–	+	+++	+++	++	+++	++	+	–
KT44	*L. paracasei*	–	+	+++	+++	++	+++	+++	++	++
KT45	*L. plantarum*	+	+++	+++	+++	++	+++	+++	+++	+++
KT51	*L. brevis*	–	+	+++	+++	++	+++	++	++	–
KT62	*L. paracasei*	–	+	+++	+++	++	+++	+++	++	++
KT65	*L. paracasei*	–	+	+++	+++	++	++	–	–	+
KT68	*L. plantarum*	+	+++	+++	+++	++	+++	+++	++	+++
KT71	*L. plantarum*	–	+	+++	+++	++	+++	+++	++	–
KT85	*L. paracasei*	–	+++	+++	+++	++	+++	+++	++	+++
KT110	*L. plantarum*	+	++	+++	+++	++	+++	+++	+++	++

+: 20-30%; ++: 40-49%; +++: 50-60%; –: no growth.

Table 2 - EPS and H_2O_2 production, casein hydrolysis and hydrophobicity of *Lactobacillus* strains (n=11).

Species	Isolate No.	H_2O_2 production (µL/mL)[a]	Casein hydrolysis (zone of hydrolysis in cm)[a]	EPS production	Hydrophobicity (%)[a]
L. paracasei	KT36	1.2±0.5	1.6±0.3	+	20.2±3.1
L. brevis	KT37	0.5±0.3	1.2±0.2	+	22.9±5.5
L. paracasei	KT38	1.3±0.3	1.0±0.2	+	46.9±4.2
L. paracasei	KT39	1.5±0.4	1.6±0.1	+	48.1±0.5
L. paracasei	KT44	0.5±0.3	1.3±0.1	+	50.4±3.0
L. plantarum	KT45	2.9±0.6	1.0±02	+	55.7±2.2
L. paracasei	KT62	1.1±0.4	1.1±0.3	+	53.2±1.3
L. paracasei	KT65	0.9±0.3	1.5±0.5	+	54.3±0.6
L. plantarum	KT68	0.9±0.3	1.4±0.1	+	58.6±0.5
L. paracasei	KT85	1.0±0.2	0.8±0.2	+	45.3±2.2
L. plantarum	KT110	1.1±0.2	0.8±0.4	+	52.2±3.1

[a]: mean and standard deviation (n=3).

STYRIAK and CONKOVA, 2002). Microbial adsorption to hexadecane drops has been experimentally validated as a method to estimate qualitatively the adsorption of isolates to epithelial cells (KIELY and OLSEN, 2000). All of the *Lactobacillus* strains produced EPS, and some of the strains had high hydrophobicity values (Table 2). *L. plantarum* KT68 had the highest hydrophobicity value (58.6%) followed by *L. plantarum* KT45 (55.7%), and *L. plantarum* KT110 (52.2%).

One of the most sought after properties of probiotic LAB is the production of antimicrobial activity against pathogens. In our study, all the supernatants derived from *L. plantarum* KT45, KT69 and KT110 cultures showed antimicrobial activity against pathogens, including *B. cereus, E. coli, E. coli* O157:H7, *Salmonella* sp., and MRSA (Table 3). These supernatants also had good antimicrobial activity against all the other LAB isolates used as indicators except for *L.*

Table 3 - Inhibition zones of supernatants obtained from *Lactobacillus* strains (n=11).

Indicator strains	Inhibition zone diameter range of supernatants (mm)[a]		
	L. paracasei (n=7)	*L. plantarum* (n=3)	*L. brevis* (n=1)
Escherichia coli (LMG 8223) [b]	–	8–12	–
E. coli O157:H7 (ATCC 35150) [c]	–	8–23	–
Bacillus subtilis (NRRL 744) [d]	–	12	–
B. cereus (LMG 8821)	12	8–15	–
Salmonella sp. (clinical isolate) [e]	10–15	8–14	10
Listeria monocytogenes (LMG 13305)	–	–	–
Enterococcus faecium (NRRL 2354)	–	–	–
Staphylococcus aureus (ATCC 25923)	–	–	–
MRSA (clinical isolate) [e]	–	19	–
VRE (clinical isolate) [e]	–	–	–
Lactobacillus bulgaricus (NRRL 548)	17	16	–
L. plantarum (NRRL 4560)	10	19	–
L. paracasei (NRRL 4562)	8–10	25	–
L. casei (NRRL 1922)	–	17	–
L. paramesenteroides (NNRL 3252)	11–15	18	15
L. buchneri (NRRL 1837)	8–18	8	–
L. curvatus (NRRL 4562)	9–15	18	–
L. rhamnosus (NRRL 442)	8–14	–	–
Lactococcus lactis ssp. *lactis* (NRRL 633)	–	19	–

–: no activity.
[a]Strains that have antimicrobial activity against at least one indicator strain indicated in the table. The averages (n=3) of the inhibition zones each active strains are given.
[b]BCCM/LMG: Bacteria Collection. Laboratorium Voor Microbiologie-Universiteit Gent, Gent-Belgium.
[c]ATCC: American Type Culture Collection, Manassas, VA 20110-2209, USA.
[d]NRRL: Agricultural Research Service, Culture Collection, Peoria. IL 61604, USA.
[e]Obtained from University of Eskisehir Osmangazi, Faculty of Medicine, Eskisehir-Turkey.

rhamnosus. The antimicrobial spectrum of the *L. paracasei* strains was relatively narrow; they inhibited only *B. cereus* and *Salmonella* sp. Similarly, culture supernatants of *L. brevis* KT37 only inhibited two test bacteria (*Salmonella* sp. and *L. paramesenteroides*). *Lactobacillus* strains that inhibited Gram-negative indicator test bacteria may be effective in reducing the urease activity in the large intestine by inhibiting Gram-negative bacteria.

After being treated with enzymes, including proteinase K, trypsin, α-chymotrypsin, lipase, and α-amylase (1 mg/mL), the activity of the culture supernatants (derivied from 11 strains) was again tested on some indicator bacteria. All the culture supernatants that were treated with proteinase K, trypsin, or α-chymotrypsin lost their antimicrobial activity, indicating that the antimicrobial activity in the supernatants is most likely the result of peptide or protein-derived compounds. Additionally, lipase or α-amylase treatment did not affect the antimicrobial activity of the supernatants, indicating that the antimicrobial effect did not stem from lipid or carbohydrate moieties.

When considering the safety of probiotics, their sensitivity to commonly used antibiotics is quite important because potential probiotics may harbor antibiotic-resistant genes that can be transferred to pathogenic bacteria in the host. Thus, the resistance of potential probiotic isolates to various therapeutic antibiotics was determined in our study (Table 4). With the exception of *L. paracasei* KT62 and KT85, all of our potential probiotic isolates were sensitive to all the protein synthesis inhibitor antibiotics we used in our experiments. Additionally, all of the strains were resistant to nalidixic acid, which is a nucleic acid synthesis inhibitor. Among 11 strains, 6 strains were resistant to vancomycin, but all of the strains were sensitive to other cell wall synthesis inhibitor antibiotics tested. Vancomycin resistance in the lactobacilli is commonly encountered. In our study, only 5 *Lactobacillus* strains were found to be sensitive to vancomycin. This result was most likely related to the high concentration of the vancomycin (30 μg/disk) used in this study. Although the minimum inhibitory concentrations (MIC) are defined for clinically important microorganisms, internationally valid MIC breakpoints or inhibition zone breakpoints for lactobacilli have not been determined yet. Therefore, we could not compare our susceptibility test results to the Clinical and Laboratory Standards Institute (CLSI, 2011) or European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2013) criteria.

Of the many advantages of fermented milk products, the most well known is their ability to counteract lactose intolerance (BRUNSER *et al.*, 2007). To determine the carbohydrate fermentation profile of our *Lactobacillus* spp., API CH50

Table 4 - Results of antibiotic susceptibility tests of *Lactobacillus* strains (n=11).

	Zones of inhibition of antibiotics (in mm)									
	Protein synthesis inhibitors						Cell wall synthesis inhibitors			Nucleic acid synthesis inhibitor
Isolate No.	Streptomycin (10 μg/disk)	Erythromycin (15 μg/disk)	Gentamicin (10 μg/disk)	Chloramphenicol (30 μg/disk)	Tetracicline (30 μg/disk)	Rifampicin (5 μg/disk)	Amoxicillin (25 μg/disk)	Penicillin G (10 μg/disk)	Vancomycin (30 μg/disk)	Nalidixic acid (30 μg/disk)
KT36	10±2*	8±2	12±2	20±3	24±2	20±3	40±2	28±2	24±3*	R
KT37	10±1	10±2	10±3	26±3	20±3	21±4	36±1	22±3	20±2	R
KT38	8±1	5±1	8±3	21±2	18±3	22±3	32±1	22±3	18±2*	R
KT39	10±2	5±2	13±2	22±4	20±2	19±2	34±1	18±2	20±2	R
KT44	10±1	18±2	19±2*	23±3	18±5	18±1	31±2	23±2	22±2*	R
KT45	8±1	10±3	10±2	21±2	20±5	24±1	30±1	20±3	24±3	R
KT62	R	16±2	8±2*	24±4	14±3	18±1	30±2	16±4	R	R
KT65	12±3*	20±3	10±3	26±3	22±3	28±3	30±1	24±3	24±2*	R
KT68	10±2	11±3	12±3	22±2	21±2	27±2	32±1	24±3	24±4	R
KT85	R	20±3	20±2	22±2	24±1	20±1	34±3	20±2	R	R
KT110	10±2	12±1	6±2	26±3	25±1	16±2	29±2	21±2	26±4	R

R: resistant.
*: colony growth observed in the inhibition zone.

test kits were used (Table 5). These tests showed that all of the isolates were capable of metabolizing numerous sugars. Except *L. paracasei* KT36 and *L. plantarum* KT110, all the strains fermented lactose, or in other words, they had β-galactosidase activity (Table 6).

Prebiotics represent an energy source that may be metabolized by the intestinal or probiotic microbiota (TOMASIK, 2003). The fermentation of prebiotics that cannot be digested by the host but can be fermented by bacteria in the large intestine yields various fermentation products, predominantly short chain fatty acids (SCFA). These SCFA create an acidic environment that can stimulate the proliferation of probiotic bacteria in the large intestine (ROBERFROID, 2007; MACFARLANE *et al.*, 2006). Fructooligosaccharides (FOS), including inulin, and galactooligo-

Table 5 - Carbohydrate fermentation profiles of *Lactobacillus* strains (n=11).

	Isolate No.										
Carbohydrates	**KT 36**	**KT 37**	**KT 38**	**KT 39**	**KT 44**	**KT 45**	**KT 62**	**KT 65**	**KT 68**	**KT 85**	**KT 110**
1. Glycerol	–	–	–	–	–	–	–	–	–	–	–
2. Erythritol	–	–	–	–	–	–	–	–	–	–	–
3. D-Arabinose	–	–	–	–	–	–	–	–	–	–	–
4. L-Arabinose	–	+	+	+	+	+	+	+	–	+	–
5. D-Ribose	+	+	+	+	+	+	+	+	+	+	+
6. D-Xylose	–	+	+	+	+	+	+	+	–	+	–
7. L-Xylose	–	–	–	–	–	–	–	–	–	–	–
8. D-Adonitol	–	–	–	–	–	–	–	–	–	–	–
9. Inositol	–	–	–	–	–	–	–	–	–	–	–
10. D-Galactose	+	+	+	+	–	+	+	+	+	–	+
11. D-Glucose	+	–	+	+	–	+	+	+	+	–	+
12. D-Fructose	+	–	+	+	–	+	+	+	+	–	+
13. D-Mannose	+	–	+	+	–	+	+	+	+	–	+
14. L-Sorbose	–	–	–	–	–	–	–	–	–	–	–
15. L-Rhamnose	+	–	–	+	–	–	–	+	–	–	–
16. Dulcitol	–	–	–	–	–	–	–	–	–	–	–
17. Salicin	–	–	–	–	–	–	–	–	–	–	–
18. D-Cellobiose	+	–	+	+	–	+	+	+	+	–	+
19. D-Maltose	+	–	+	+	–	+	+	+	+	–	+
20. D-Lactose	–	+	+	+	+	+	+	+	+	+	–
21. D-Melibiose	–	–	–	–	–	–	–	+	–	–	–
22. D-Saccharose	+	–	+	+	–	+	+	+	+	–	+
23. D-Trehalose	–	–	+	+	–	+	+	+	+	–	+
24. Inulin	+	–	+	+	–	+	+	+	+	–	+
25. D-Melezitose	+	–	+	+	–	+	+	+	+	–	+
26. D-Raffinose	+	–	+	+	–	+	+	+	+	–	+
27. Amidon (starch)	+	–	+	+	–	+	+	+	+	–	+
28. Glycogen	–	+	+	+	–	+	+	+	+	+	+
29. Xylitol	+	–	+	+	–	+	+	+	+	–	+
30. Gentiobiose	–	+	+	+	–	+	+	+	+	+	+
31. D-Turanose	+	+	+	+	–	+	+	+	+	–	+
32. D-lyxose	+	–	+	+	–	+	+	+	+	–	+
33. D-Tagatose	+	–	–	–	–	–	–	–	–	–	–
34. Esculin (ferric citrate)	+	–	+	+	–	+	+	+	+	–	–
35. Methyl-α D-Mannopyranoside	–	–	–	–	–	+	–	+	+	–	+
36. Methyl-α D-Glucopyranoside	–	–	–	–	–	–	–	–	–	–	–
37. N-Acetylglucosamine	–	–	–	–	–	–	–	–	–	–	–
38. Amygdalin	–	–	–	–	–	–	–	–	–	–	–
39. Arbutin	+	–	–	–	–	–	–	+	–	–	–
40. D-Arabitol	+	–	+	+	–	+	+	+	+	–	–
41. L- Arabitol	–	–	–	–	–	–	–	–	–	–	–
42. Potassium Gluconate	+	–	+	+	–	+	+	+	+	–	–
43. Potassium 2-Ketogluconate	–	–	–	–	–	–	–	–	–	–	–
44. Potassium 5- Ketogluconate	–	–	–	–	–	–	–	–	–	–	–
45. D-Mannitol	–	–	–	–	–	–	–	–	–	–	–
46. D-Sorbitol	–	–	–	–	–	–	–	–	–	–	–
47. D-Fucose	–	–	–	–	–	–	–	–	–	–	–
48. L-Fucose	–	–	–	–	–	–	–	–	–	–	–
49. Methyl-βD-Xylopyranoside	–	–	–	–	–	–	–	–	–	–	–

+: fermented; –: not fermented.

Table 6 - Enzymatic profiles of *Lactobacillus* strains (n=11).

Enzymes	Isolate No.										
	KT 36	KT 37	KT 38	KT 39	KT 44	KT 45	KT 62	KT 65	KT 68	KT 85	KT 110
1. Alkaline phosphatase	–	–	–	–	+	–	–	–	–	–	–
2. Esterase	–	+	+	–	+	–	+	+	+	–	–
3. Esterase lipase	–	+	+	–	+	–	+	+	+	+	+
4. Lipase	–	–	–	–	–	–	–	–	–	–	–
5. Leucine arylamidase	+	+	+	+	+	+	+	+	+	+	+
6. Valine arylamidase	+	+	+	+	+	+	+	+	+	+	–
7. Cystine arylamidase	–	–	–	+	+	–	–	–	–	+	+
8. Trypsin	–	–	–	–	–	–	–	–	–	–	–
9. α-Chymotrypsin	–	–	–	–	–	–	–	–	–	–	–
10. Acid phosphatase	–	+	+	+	+	–	+	+	+	+	–
11. Naphthol-AS-BI-phosphohydrolase	+	+	+	+	+	+	+	+	+	+	+
12. α-galactosidase	–	+	+	+	–	–	+	+	+	+	–
13. β-galactosidase	–	+	+	+	+	+	+	+	+	+	–
14. β-glucuronidase	–	+	+	–	–	–	+	+	+	+	–
15. α-glucosidase	–	+	+	+	+	+	+	+	+	+	–
16. β-glucosidase	+	+	+	+	+	+	+	+	+	+	–
17. *N*-acetyl-β-glucosaminidase	–	–	+	–	+	+	+	+	–	–	–
18. α-mannosidase	–	–	–	–	–	–	–	–	–	–	–
19. α-fucosidase	–	–	–	–	–	–	–	–	–	–	–

+: enzyme activity present; -: no activity.

saccharides (GOS) have such a prebiotic effect (SWENNEN *et al.*, 2006). Another reported advantage of an acidic environment in the large intestine is the inhibition of some Gram-negative bacteria, especially those with urease enzymatic activity. Antibiotics are currently being used to treat HE to prevent the urease enzymatic activity of Gram-negative bacteria in the large intestine (SOLGA, 2003). In our study, *Lactobacillus* strains had the ability to ferment a broad spectrum of sugars (Table 5) including inulin. Therefore, inulin could be tested as a prebiotic in probiotic food formulations used with our promising probiotic strains.

CONCLUSIONS

Kargi tulum cheese is an artisanal cheese produced by traditional methods without the addition of starter cultures in small local dairies in Çorum-Turkey. To the best of our knowledge, there is no previous study of the probiotic characteristics of its natural fermentative microflora. In our study, we found that the natural fermentative microflora of Kargi tulum cheese contains probiotic members. *L. plantarum* KT45, KT68, and KT110 and *L. paracasei* KT39 exhibited many of the desired probiotic characteristics. These strains are resistant to bile, GJ and extreme acidity, able to produce bacteriocins, EPS and H_2O_2, sensitive to several antibiotics used in treating diseases, and have the potential to adhere to epithelial cells. Therefore, it can be concluded that these strains warrant further examination, including *in vivo* tests. Additionally, more detailed research is needed on the bacteriocins produced by our *Lactobacillus* strains that have antimicrobial effects on food-borne and clinical pathogens.

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PAPER

PHENOLIC PROFILE IN SELECTED SICILIAN WINES PRODUCED BY DIFFERENT TECHNIQUES OF BREEDING AND CROPPING METHODS

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ABSTRACT

The phenolic profiles of selected Sicilian wines, produced from grapes that differed from one another in techniques of breeding and cropping methods, were investigated. Phenolics were identified in Sicilian young red wines from autochthonous and allochthonous grapes by a rapid HPLC-DAD/MS method. The effect of cultivation techniques used in vineyard on the phenolic composition was confirmed. This study allowed verifying such dependence considering three techniques of breeding and two horticultural cropping methods. In order to detect tendencies in the analysed samples according to the phenolic compound contents, Principal Components Analysis (PCA) and Canonical Discriminant Analysis (CDA) were used.

- Keywords: food analysis, grape, HPLC-MS, phenol, vineyard management, statistical analysis -

INTRODUCTION

Current oenological research is oriented at defining wine quality in relationship to its chemical composition, pharmacological properties and safety; in fact, some wine micro-constituents, such as phenols, are known as healthy compounds.

Phenolic compounds constitute one of the most important quality parameters of wines since they contribute to their organoleptic characteristics, particularly colour, astringency, and bitterness. Wine phenolics belong to two main groups, non-flavonoid (hydroxybenzoic and hydroxycinnamic acids and their derivatives, stilbenes and phenolic alcohols) and flavonoid (anthocyanins, flavanols, flavonols and dihydroflavonols) compounds. Anthocyanins are the main phenolic compounds involved in the colour of red wines. The astringency and bitterness of young wines is mainly due to phenolic acids and flavanols (RIBICHAUD and NOBLE, 1990). Hydroxycinnamic acids and flavanols, together with flavonols, act as co-pigments of anthocyanins (EIRO and HEINONEN, 2002). Phenolic compounds, especially flavonoids and stilbenes, have been recognized as responsible for several beneficial physiological effects associated with red wine consumption, mostly due to their antioxidant and anti-inflammatory properties (STOCLET *et al.*, 1999). However, despite much interest in the antioxidant activity of red wine, it is uncertain which of phenols exhibit the greatest antioxidant effect (KERRY and ABBEY, 1997).

In recent years, numerous research studies affirmed the moderate consumption of wine produces beneficent effects to prevent cardiovascular diseases, certain types of cancer and other diseases related to aging (FRIEDMAN and KIMBAL, 1986; SCALBERT *et al.*, 2005). It has been hypothesized that the phenolic substances of wine might be responsible for these preventive beneficent effects on degenerative illnesses as those cardiovascular, inflammatory, cancer, Alzheimer and ageing. The great advantage of wine as a matrix for phenols in the diet is that in wines they are present in the soluble state and are hence more biologically available, in contrast with plant foods that contain their phenol compounds in polymeric, insoluble or strongly bonded forms, and are thus less available for absorption (BORBALÁN *et al.*, 2003).

Wine may have very complex phenolic composition and contents that are affected by several factors such as grapes variety, ripening stage, climate, soil, place of growing and vine cultivation (KLAUTSKY and ARMSTRONG, 1993). In addition, winemaking processes and reactions that take place during maturation also modify it. Not all the phenolic compounds have the same biological activity (ARNOUS *et al.*, 2001), and the phenolic composition of the wines can be conditioned both from environmental factors and from technological treatments (VILLAÑO *et al.*, 2006). Besides, it was verified that the natural antioxidants decrease on account of the treatments and during the maintenance (SCHWARZ *et al.*, 2004). Moreover, experimental evidences have even shown that, in some cases, the technological treatments are able to influence the content of compounds with antioxidant properties (IVANOVA *et al.*, 2011).

Phenolic compounds greatly contribute to the composition, the microbial stability, the physical-chemical and sensory qualities of all the wines (RIBÉREAU-GAYON *et al.*, 1982). During vinification, alcoholic fermentation and aging, these compounds gradually modified owing to reaction among anthocyanins, flavan-3-ol, proanthocyanidins and other compounds such as flavonols themselves (FULCRAND *et al.*, 1998).

Owing to an important role of phenolic compounds in determining wine quality, a great number of studies have been done on the effects of climate factors, soil type, vine maturity, oenological techniques (LORRAIN *et al.*, 2011). The phenolic composition of wines has yielded extensive literature, especially for wines produced in Spain, France and Greece. Particularly for the wines produced in Sicily, to the best of our knowledge, apart from few report (DUGO *et al.*, 2000; LA TORRE *et al.*, 2006), the analytical polyphenolic composition is largely unknown.

Sicily is considered one of the oldest European viticultural regions, and with its warm temperatures, hilly terrain, sea breezes and rich soil, it has more area under vine production than any other major winemaking region in Italy. Sicily produces at least 17% of all the Italian wines, most of which are now protected by Controlled Denomination of Origin (DOC) regulation. In recent years, wine has been one of the few Sicilian agricultural products that has seen a rise in export levels, both in quality and average unit value compared to previous years (CRESCIMANNO *et al.*, 2002). Among the Sicilian white wines, the most distinctive ones are Insolia, Damaschino or Grillo, sometimes blended with Chardonnay. Among the red wines, the most diffused native varieties are Nero d'Avola and Nerello Mascalese that are vinified in their purest varietal form or blended with allochthonous varieties such as Cabernet Sauvignon, Merlot and Syrah. Particularly, the variety Nero d'Avola is the most diffused in the island and wines are widely commercialised on the national market.

Considering that wineries usually rely on global chemical determinations, together with sensory analysis, to make decisions concerning product quality improvements, the aim of the present work was to verify the influence of three different techniques of breeding and two different horticultural cropping methods on the phenolic composition of selected wines. We chose four historical varieties that, thanks to the energies and experience of the vine-dressers, have a good

chance of not succumbing to the wave of flattening that threatens the production from time, removing the viticulture and cultural jewels of Italian heritage.

For that purpose, red wines from autochthonous *vitis vinifera* var. Nero d'Avola, Nerello Mascalese, Frappato and Perricone grown in Sicily, have been analyzed. This study included also red wine cultivars Merlot, Petit Verdot, Cabernet Sauvignon and Cabernet Franc that are grown world-wide and that are among the main allochthonous cultivars in Sicily. In this study, HPLC-DAD coupled with ESI-MS technique and spectrophotometric assays (LA TORRE *et al.*, 2006) have been applied for analysis of wines obtained from grapes of vines under different techniques of breeding horticultural cropping methods. The data obtained for the investigated varieties have been compared evaluating the influence of the varietal factor and the different treatment in the vineyard.

MATERIALS AND METHODS

Chemicals

The solvent used for HPLC-MS analyses (CH_3CN, H_2O and formic acid) were HPLC grade (from Sigma-Aldrich; Milan, Italy) and were degasified in an ultrasound bath before analyses.

Polyphenolic standards tested included: (-)-epicatechin, (+)-catechin, gallic acid, 3,4dihydroxybenzoic acid (protocatechuic acid), 4-hydroxy-3-methoxybenzoic acid (vanillic acid), 4hydroxy3,5dimethoxybenzoic acid (syringic acid), 3,4-dihydroxycinnamic acid (caffeic acid), 4hydroxy-3-methoxycinnamic acid (ferulic acid), 4-hydroxycinnamic acid (*p*-coumaric acid), tyrosol (2-(4-hydroxyphenil) ethyl alcohol) and *trans*-resveratrol (Sigma-Aldrich, Milan, Italy).

The other phenolic compounds were procyanidin B1, procyanidin B2, ethylgallate, quercetin, isoquercitrin (quercetin-3-O-glucoside), kaempferol, kaempferol 3-O-glucoside, isorhamnetin, isorhamnetin 3-O-glucoside, rutin (quercetin-3-O-rutinoside), myricetin and were purchased from Extrasynthese (Genay, France).

The standards were dissolved in aqueous formic acid (pH=3)/methanol (90:10), and injected to determine individual retention times. The stock solution of the individual standards were prepared by dissolving 100 mg standard into 100 mL aqueous formic acid (pH=3)/methanol (90:10). The stock solutions of the standards were diluted to obtain five different concentrations. These solutions were injected for linearity range and detection limit tests.

The stock solution of *cis*-resveratrol was produced by UV irradiation of *trans*-resveratrol in methanol for 120 min at 366 nm (TRELA and WATERHOUSE, 1996). A five points calibration curve for the quantitative analysis of *cis*-resveratrol was obtained as described by Romero-Pérez (ROMERO-PÉREZ *et al.*, 1996).

Resveratrol glucosides were quantified after enzymatic hydrolyses with β-glucosidase at 50°C (LA TORRE *et al.*, 2004).

All the solutions were filtered through a 0.45 µm glass-microfiber GMF Whatman chromatographic filter, before the HPLC analysis, and mobile phase solvents were degassed before use.

Samples

The study was carried out on wine samples that were elaborated from four autochthonous *vitis vinifera* variety (Nero d'Avola, Frappato, Nerello Mascalese and Perricone) and four allochthonous *vitis vinifera* variety (Merlot, Petit Verdot, Cabernet Sauvignon and Cabernet Franc) harvested in the 2011 vintage in Sicily. Each sample was obtained from the most representatives red varieties of autochthonous and allochthonous Sicilian grapes and the samples include most of the wine cultivars that are commercially available.

All the plants were cultivated in level grounds (200 m asl) located in five different Sicilian provinces (Trapani, Ragusa, Agrigento, Siracusa and Catania). The wines of var. Nero d'Avola, Frappato and Cabernet Sauvignon were three for each cultivar and differed for techniques of breeding, while the wines of var. Nerello Mascalese, Perricone, Cabernet Franc and Petit Verdot were two for each cultivar and differed for horticultural cropping methods only. The wines of var. Merlot were obtained both according different techniques of breeding and horticultural cropping methods. The vines of var. Nero d'Avola, Frappato, Cabernet Sauvignon and Merlot were maintained under three different techniques of breeding (cortina: hanging down training system; controspalliera: vertical-trellised training system; alberello: the plant is scaffolding at a height varying from 20 to 30 cm and ends with a system of three or four short branches, bearing spurs cut in one or three buds, and sometimes, fruit), while the vines of var. Nerello Mascalese, Perricone, Cabernet Franc, Petit Verdot and Merlot were bred alberello and kept under two different horticultural cropping methods (with pruning and without pruning of bunch of grapes). The vines of the same variety had the same age; the grapes of this study were manually harvested at maturity but harvest time was made accordingly to the different variety grapes; detail of the grapes and musts analysed are given in Table 1a,b. All the grapes were transported to the same wineproduction centre and were processed separately in the same way.

Vinification

To eliminate the influence of the vinification procedure and to guarantee that the results only

Table 1a) - Details on grapes and musts analysed.

Wine	Grape	Comment
1	Nero d'Avola	- Grapes grown cortina (CRT) in Siracusa - The must was obtained from 750 quintals of grapes
2	Nero d'Avola	- Grapes grown alberello (ALB) in Siracusa - The must was obtained from 750 quintals of grapes
3	Nero d'Avola	- Grapes grown controspalliera (CSP) in Siracusa - The must was obtained from 550 quintals of grapes
4	Frappato	- Grapes grown cortina (CRT) in Ragusa - The must was obtained from 300 quintals of grapes
5	Frappato	- Grapes grown alberello (ALB) in Ragusa - The must was obtained from 300 quintals of grapes
6	Frappato	- Grapes grown controspalliera (CSP) in Ragusa - The must was obtained from 300 quintals of grapes
7	Nerello Mascalese	- Grape cropped DRD in Catania - The must was obtained from 300 quintals of grapes
8	Nerello Mascalese	- Grape cropped LED in Catania - The must was obtained from 300 quintals of grapes
9	Perricone	- Grape cropped DRD in Agrigento - The must was obtained from 300 quintals of grapes
10	Perricone	- Grape cropped LED in Agrigento - The must was obtained from 300 quintals of grapes
11	Cabernet Sauvignon	- Grapes grown cortina (CRT) in Trapani - The must was obtained from 450 quintals of grapes
12	Cabernet Sauvignon	- Grapes grown alberello (ALB) in Trapani - The must was obtained from 450 quintals of grapes
13	Cabernet Sauvignon	- Grapes grown controspalliera (CSP) in Trapani - The must was obtained from 450 quintals of grapes
14	Merlot	- Grapes grown cortina (CRT) in Trapani - The must was obtained from 500 quintals of grapes
15	Merlot	- Grapes grown alberello (ALB) in Trapani - The must was obtained from 500 quintals of grapes
16	Merlot	- Grapes grown controspalliera (CSP) in Trapani - The must was obtained from 500 quintals of grapes
17	Merlot	- Grape cropped DRD in Trapani - The must was obtained from 350 quintals of grapes
18	Merlot	- Grape cropped LED in Trapani - The must was obtained from 350 quintals of grapes
19	Cabernet Franc	- Grape cropped DRD in Agrigento - The must was obtained from 300 quintals of grapes
20	Cabernet Franc	- Grape cropped LED in Agrigento - The must was obtained from 300 quintals of grapes
21	Petit Verdot	- Grape cropped DRD in Ragusa - The must was obtained from 300 quintals of grapes
22	Petit Verdot	- Grape cropped LED in Ragusa - The must was obtained from 300 quintals of grapes

Table 1b) - Details on grapes and musts analysed.

Must of grape	Agronomic technique	Sugar concentration (g/L)	Titrable acidity (g/L of tartaric acid)	pH
Nero d'Avola	CRT	170.2	11.43	2.99
Nero d'Avola	ALB	176.7	9.43	3.16
Nero d'Avola	CSP	185.8	9.21	3.17
Frappato	CRT	200.4	8.97	3.54
Frappato	ALB	211.3	8.38	3.35
Frappato	CSP	203.5	8.21	3.41
Nerello Mascalese	DRD	200.5	7.91	3.87
Nerello Mascalese	LED	178.5	8.08	3.69
Perricone	DRD	178.5	8.37	3.31
Perricone	LED	205.3	8.21	3.35
Cabernet Sauvignon	CRT	201.4	7.88	3.31
Cabernet Sauvignon	ALB	231.3	8.60	3.37
Cabernet Sauvignon	CSP	205.3	7.40	3.29
Merlot	CRT	214.4	5.16	3.58
Merlot	ALB	210.5	6.14	3.53
Merlot	CSP	214.3	5.34	3.64
Merlot	DRD	224.8	5.64	3.65
Merlot	LED	223.5	6.34	3.52
Cabernet Franc	DRD	201.4	6.90	3.31
Cabernet Franc	LED	184.5	6.88	3.35
Petit Verdot	DRD	167.6	10.06	3.18
Petit Verdot	LED	198.8	10.40	3.11

reflect the agronomic choices, the condition of vinification was the same for all the grape varieties. Moreover, to reduce the influence of the fermentation conditions and to guarantee that the results are the consequences of the agronomic technique, three independent fermentation processes were investigated.

The vinification was carried out from a well-known Sicilian wine industry. Newly cropped *vitis vinifera* were crushed and destemmed, then spiked with SO_2 (5 g/hL) and selected commercial yeasts (30 g/hL) (*Saccharomyces cereviseae* phylum *Ellipsoideus*, FERMOL MEDITERRANÉE) provided by AEB Spa (Brescia, Italy) and macerated in a 25,000-L stainless-steel vat in contact with the marcs for 10 days at 28°C, effecting three fullings a day. The wine was drawn from the vat and the grape wastes were crushed by hydraulic press. In all cases, the malolactic fermentation was spontaneous and fully completed, which was facilitated by the low levels of sulphur dioxide used (GONZÀLEZ-NEVES *et al.*, 2004).

After 4 weeks, the lees were removed and the wine was spiked with SO_2 (2-3 g/hL) and bottled.

Each sample was separately vinified in triplicates, following the protocol mentioned above. All wines were not aged after alcoholic and malolactic fermentations and fresh wines of about 1,000 mL were sampled.

After respectively sampling, each sample was divided into three parts and then stored in the dark at -20°C before analyses. The analyses were carried out without any prior purification of the sample; the wine samples were filtered through a 0.45 µm glassmicrofiber GMF Whatman chromatographic filter, before the HPLC analysis.

Analysis of phenolics by HPLC-MS

The determination of phenolics was carried out using a direct injection chromatographic method (LA TORRE *et al.*, 2006). The analyses were performed by a Shimadzu system equipped with two pumps LC10-A, a controller SCL-10A, with a photodiode array detector SPD M10Avp equipped with a semi microcell and operating at wavelengths between 200 and 600 nm. The system was coupled with a MS detector Shimadzu 2010 with an ESI interface.

The separation was performed through a C18 Supelco Discovery column (15 cm x 2.1 mm I.D., 5 µm particle size), protected by a Supelco guard column packed with the same stationary phase. The mobile phase was a gradient consisting of water (pH=3 by formic acid, solvent A) and acetonitrile (pH=3 by formic acid, solvent B): 0.01-20.00 min 5% B isocratic; 20.01-50.00 min, 5-40% B; 50.01-55.00 min, 40-95% B; 55.01-60.00 min 95 % B isocratic. The flow rate was 0.2 mL/min and the analyses were performed at 20°C. The samples were directly injected into the column using a Rheodyne (model 7725i) injection valve.

Negative-ion mode electrospray ionization mass spectrometry (ESI-MS) spectra were recorded using the following condition: probe high voltage, 4 kV; nebulizing gas (N_2) flow rate, 4.5 L/min; curved desolvation line (CDL) voltage, 10 V; CDL temperature, 250°C; acquisition mode, SCAN, 50-700 *m/z* and deflector voltage at -20 and -80 V. The SIM (selected ion monitoring) mode was used when a search for some particular ions should be done. UV and MS data were acquired and processed using operating system Windows NT 4.0.

Statistical analysis

The results obtained from the analysis of phenolic compounds in different wine samples were submitted to statistical elaboration using the SPSS 13.0 software package for Windows (SPSS Inc., Chicago, IL). Three data matrices were built to establish relations between samples and phenolic compounds. The first matrix, wherein rows are the 22 different wines analyzed and wherein columns are the 29 variables, was used in order to investigate the differences between autochthonous and allochthonous samples according to the phenol content. The data were subdivided into two groups: the first (10 cases) consisting of samples obtained from autochthonous vines and the second (22 cases) obtained from allochthonous vines. The second matrix (10x29) was exploited to describe the distribution data of each phenolic compound analyzed in wine samples from autochthonous vines: the cases are divided into four groups in agreement to autochthonous *vitis vinifera* variety (Nero d'Avola, Frappato, Nerello Mascalese and Perricone). The third matrix (12x29) was constructed with the phenolic compounds of wines obtained from allochthonous vines, wherein the cases are divided into four groups according to allochthonous variety of *vitis vinifera* (Merlot, Petit Verdot, Cabernet Sauvignon and Cabernet Franc). It was used to check differences among wines obtained from allochthonous vines. Initially, the non-parametric Mann-Whitney U or Kruskal Wallis tests were applied to study the significance of differences. Successively, data sets were normalized in order to achieve independence on the scale factors of the different variables and Factor Analysis with Principal Components extraction were performed to decrease the number of descriptors retaining the maximum amount of variability present in the experimental data. Finally, Canonical Discriminant Analysis (CDA) was further conducted to classify different wine samples.

RESULTS AND DISCUSSION

The polyphenols included in this study were selected based on their molecular weight and for the reason that, even if they are pre-

sent in low amounts in wines, however they play important role in influence their sensory qualities (REVILLA and GONZÁLEZ-SANJOSÉ, 2003). In particular, hydroxycinnamic acids and flavonols, together with flavan-3-ols act as copigments of anthocyanins, participating in the colour stabilization of red wine (BOULTON, 2001; PUÉRTOLAS *et al.*, 2009). Sensorial properties such as astringency, bitterness and structure of young red wines are largely due to monomeric flavan-3-ols, proanthocyanidins and phenolic acids (SINGLETON and ESSAU, 1969; FISCHER and NOBLE, 1994; GAWEL, 1998; SARNI-MANCHADO *et al*, 1999). Among the phenolic compounds, flavonoids and stilbenes have been recognized as the mainly responsible compounds for the beneficial physiological effects associated with red wine consumption (ESTRUCH, 2000). Furthermore, it has been reported that the interaction between quercetin and resveratrol plays a significant role in the health effects of red wine, especially with respect to their effect on the inhibition of the inducible nitric oxide synthase pathway (MAN-YING CHAN *et al.*, 2000).

In all the samples analysed, 25 phenolic compounds were clearly identified according to their retention time compared with those of the phenols tested, as well as their spectral characteristics (LA TORRE *et al.*, 2006). The quantitative study was performed, for all the samples, at wavelengths that show more sensitivity to each phenolic compound and was carried out directly, by the external standard method, interpolating the peak area in the correspondent linear calibration curve. A calibration curve for each phenolic compound was obtained by analyzing the standard solutions diluted at different concentrations. These standard solutions, obtained from stock solutions, were injected for the linearity range and detection limit tests. In this study, each analysis was carried out in triplicate and each value present in the Table 2,3 is the mean of nine data + 95% confidence interval. The wines were grouped according to grape variety.

Table 2 - Occurrence of phenolic compounds in Sicilian wines from a) autochthonous grapes of *Vitis vinifera* var. *Nero d'Avola* and *Frappato* and b) allochthonous grapes of *Vitis vinifera* var. *Cabernet Sauvignon* and *Merlot* maintained under three different techniques of breeding (*cortina* = CRT. *controspalliera* = CSP and *alberello* = ALB). Values are expressed as mean values of nine data (mg/L) ± 95 % confidence interval (*n=9*).

a)	Nero d'Avola			Frappato		
Compound	**ALB**	**CSP**	**CRT**	**ALB**	**CSP**	**CRT**
Gallic Acid	44.24 ± 1.43	41.83 ± 1.40	46.91 ± 1.55	30.50 ± 1.02	29.92 ± 0.97	38.74 ± 0.12
Protocatechuic Acid	2.54 ± 0.11	3.00 ± 0.13	3.15 ± 0.14	2.51 ± 0.11	2.31 ± 0.10	2.78 ± 3.13
Tyrosol	44.63 ± 3.88	22.33 ± 1.83	46.28 ± 3.73	36.66 ± 3.00	33.12 ± 2.88	38.81 ± 0.37
Vanillic Acid	7.22 ± 0.63	6.79 ± 0.62	7.29 ± 0.61	4.45 ± 0.41	4.37 ± 0.38	4.49 ± 0.26
Syringic Acid	3.77 ± 0.34	2.69 ± 0.23	3.48 ± 0.30	2.81 ± 0.24	2.77 ± 0.25	2.99 ± 0.27
Caffeic Acid	3.86 ± 0.29	3.97 ± 0.29	6.43 ± 0.49	2.74 ± 0.2	2.7 ± 0.20	3.46 ± 0.02
Ferulic Acid	0.45 ± 0.02	0.57 ± 0.02	0.65 ± 0.02	0.42 ± 0.02	0.49 ± 0.02	0.58 ± 0.02
p-Coumaric Acid	0.41 ± 0.02	0.44 ± 0.02	0.68 ± 0.03	0.50 ± 0.03	0.37 ± 0.02	0.33 ± 1.89
Procyanidin B 1	42.06 ± 1.88	58.91 ± 2.49	62.35 ± 2.86	39.3 ± 1.66	40.00 ± 1.79	41.12 ± 0.48
Procyanidin B 2	18.07 ± 0.67	22.41 ± 0.83	23.59 ± 0.94	6.94 ± 0.26	9.84 ± 0.37	12.11 ± 1.24
(±)-Catechin	50.21 ± 1.12	53.29 ± 1.32	64.37 ± 1.60	32.48 ± 0.81	38.93 ± 0.87	49.84 ± 0.43
(-)-Epicatechin	33.72 ± 0.59	31.95 ± 0.62	33.37 ± 0.62	28.42 ± 0.55	29.97 ± 0.52	23.29 ± 0.24
Ethyl gallate	7.31 ± 0.20	7.86 ± 0.20	7.93 ± 0.23	6.13 ± 0.15	5.74 ± 0.16	8.45 ± 0.47
Rutin	7.82 ± 0.41	18.32 ± 0.91	12.57 ± 0.64	8.70 ± 0.43	9.47 ± 0.49	9.15 ± 0.41
Isoquercitrin	10.21 ± 0.23	23.61 ± 0.59	17.45 ± 0.37	11.24 ± 0.28	13.68 ± 0.31	19.21 ± 0.03
Isorhamnetin-3-O-glucoside	1.15 ± 0.03	1.56 ± 0.04	1.35 ± 0.04	0.84 ± 0.02	1.28 ± 0.03	0.96 ± 0.07
Kaempferol-3-O-glucoside	3.71 ± 0.08	5.83 ± 0.14	6.63 ± 0.15	3.30 ± 0.08	3.14 ± 0.07	3.05 ± 0.08
Myricetin	3.37 ± 0.07	5.68 ± 0.14	4.56 ± 0.10	3.27 ± 0.08	4.78 ± 0.09	3.50 ± 0.23
Quercetin	3.12 ± 0.11	10.47 ± 0.39	6.22 ± 0.22	6.10 ± 0.23	11.62 ± 0.40	6.34 ± 0.02
Kaempferol	0.20 ± 0.01	0.45 ± 0.02	0.31 ± 0.02	0.32 ± 0.02	0.29 ± 0.02	0.43 ± 0.01
Isorhamnetin	0.14 ± 0.01	0.23 ± 0.01	0.19 ± 0.01	0.32 ± 0.02	0.16 ± 0.01	0.18 ± 0.05
trans-Resveratrol	0.89 ± 0.06	1.26 ± 0.05	1.01 ± 0.05	0.75 ± 0.04	1.42 ± 0.07	0.91 ± 0.02
cis-resveratrol	0.12 ± 0.01	0.14 ± 0.02	0.14 ± 0.02	0.23 ± 0.03	0.16 ± 0.02	0.20 ± 0.06
trans-Piceid	1.17 ± 0.07	1.92 ± 0.09	1.59 ± 0.11	0.87 ± 0.05	1.65 ± 0.10	0.98 ± 0.06
cis-Piceid	0.59 ± 0.05	1.00 ± 0.08	0.75 ± 0.06	0.78 ± 0.06	0.86 ± 0.07	0.71 ± 0.06
Total content						
Simple phenols (mg/L gallic acid)	123.31	93.28	131.64	94.15	88.36	108.10
Flavan-3-ols (mg/L catechin)	114.10	127.81	140.86	84.10	93.91	99.84
Flavonols (mg/L myricetin)	21.49	48.34	35.76	25.51	34.89	31.57
Stilbenes (mg/L resveratrol)	2.28	3.11	2.52	1.94	3.05	2.10

(continued table 2).

b)

Compound	Cabernet Sauvignon			Merlot		
	ALB	CSP	CRT	ALB	CSP	CRT
Gallic Acid	91.24 ± 2.94	83.83 ± 2.81	114.18 ± 3.77	98.30 ± 3.25	97.49 ± 3.27	120.82 ± 3.93
Protocatechuic Acid	3.69 ± 0.16	2.83 ± 0.13	2.44 ± 0.11	6.70 ± 0.30	6.79 ± 0.30	6.34 ± 0.27
Tyrosol	64.62 ± 5.62	63.96 ± 5.24	67.24 ± 5.43	82.80 ± 7.19	71.76 ± 5.79	90.07 ± 7.38
Vanillic Acid	7.40 ± 0.64	6.58 ± 0.60	9.08 ± 0.76	4.40 ± 0.40	4.84 ± 0.40	6.44 ± 0.57
Syringic Acid	4.08 ± 0.36	4.99 ± 0.42	5.17 ± 0.45	2.01 ± 0.17	2.56 ± 0.22	2.45 ± 0.22
Caffeic Acid	9.04 ± 0.67	10.93 ± 0.79	13.55 ± 1.04	7.04 ± 0.51	9.37 ± 0.70	9.32 ± 0.71
Ferulic Acid	0.94 ± 0.04	1.19 ± 0.05	1.51 ± 0.06	0.54 ± 0.04	0.64 ± 0.02	0.96 ± 0.04
p-Coumaric Acid	1.11 ± 0.06	0.93 ± 0.05	0.70 ± 0.04	1.10 ± 0.06	1.04 ± 0.05	1.00 ± 0.05
Procyanidin B 1	66.64 ± 2.98	47.16 ± 1.99	66.76 ± 3.06	58.81 ± 2.48	70.26 ± 3.14	77.10 ± 3.54
Procyanidin B 2	12.86 ± 0.48	26.25 ± 0.98	22.43 ± 0.89	20.23 ± 0.75	20.16 ± 0.75	30.67 ± 1.22
(±)-Catechin	41.91 ± 0.94	54.71 ± 1.36	78.44 ± 1.95	47.18 ± 1.05	48.68 ± 1.21	65.52 ± 1.63
(-)-Epicatechin	41.99 ± 0.73	65.13 ± 1.26	86.69 ± 1.61	60.13 ± 1.16	67.51 ± 1.17	71.55 ± 1.33
Ethyl gallate	10.54 ± 0.29	4.25 ± 0.11	8.86 ± 0.25	7.49 ± 0.21	6.03 ± 0.16	9.95 ± 0.25
Rutin	5.61 ± 0.29	10.99 ± 0.56	8.53 ± 0.42	5.39 ± 0.27	7.78 ± 0.39	6.47 ± 0.34
Isoquercitrin	6.58 ± 0.15	11.97 ± 0.30	7.22 ± 0.15	6.36 ± 0.13	5.01 ± 0.12	3.22 ± 0.07
Isorhamnetin-3-O-glucoside	0.32 ± 0.01	0.62 ± 0.02	0.60 ± 0.02	0.57 ± 0.01	0.76 ± 0.02	0.98 ± 0.02
Kaempferol-3-O-glucoside	0.15 ± 0.00	1.96 ± 0.04	2.14 ± 0.05	1.32 ± 0.03	3.20 ± 0.08	3.15 ± 0.07
Myricetin	16.01 ± 0.32	19.19 ± 0.48	18.58 ± 0.42	15.28 ± 0.34	18.79 ± 0.47	17.75 ± 0.35
Quercetin	16.55 ± 0.82	23.53 ± 0.62	17.92 ± 0.64	14.04 ± 0.51	31.21 ± 1.16	30.03 ± 1.06
Kaempferol	0.42 ± 0.02	0.34 ± 0.02	0.30 ± 0.02	0.10 ± 0.01	0.26 ± 0.01	0.42 ± 0.02
Isorhamnetin	0.50 ± 0.02	0.56 ± 0.03	0.47 ± 0.02	0.35 ± 0.02	0.65 ± 0.03	0.83 ± 0.04
trans-Resveratrol	1.75 ± 0.09	2.55 ± 0.13	1.79 ± 0.09	3.28 ± 0.17	3.88 ± 0.19	3.62 ± 0.19
Cis-resveratrol	0.84 ± 0.10	1.42 ± 0.18	1.01 ± 0.13	2.13 ± 0.26	2.73 ± 0.34	2.84 ± 0.35
trans-Piceid	0.53 ± 0.03	0.84 ± 0.05	0.65 ± 0.04	1.55 ± 0.09	2.64 ± 0.16	1.89 ± 0.11
cis-Piceid	0.43 ± 0.04	0.52 ± 0.04	0.65 ± 0.05	1.51 ± 0.13	1.50 ± 0.12	1.30 ± 0.11
Total content						
Simple phenols (mg/L gallic acid)	205.48	192.67	235.77	228.57	216.15	266.61
Flavan-3-ols (mg/L catechin)	123.78	156.67	209.83	146..96	161.56	191.14
Flavonols (mg/L myricetin)	49.50	60.63	49.57	50.49	88.37	83.70
Stilbenes (mg/L resveratrol)	3.15	4.76	3.56	7.20	9.03	8.32

In order to perform the quantization of the two resveratrol glycosides, because we did not compare the results with authentic samples since they are not commercial products, we operated an enzymatic hydrolysis with β-glycosidase (LA TORRE *et al.*, 2004). So after hydrolysis, *cis* and *trans*-resveratrol signal area increased and we determined the concentration of *cis* and *trans*-piceid subtracting the values obtained before hydrolyses to those after hydrolyses.

To better understand the influence of techniques of breeding and cropping methods on the phenolic profiling and to compare the total content of the phenolic compound studied, for each wine sample these antioxidant compounds were pooled into four parts: simple phenols (they include hydroxybenzoic acids, hydroxycinnamic acids and esters), flavan-3-ols (that are catechins and procyanidins), flavonols and stilbenes. Simple phenols were quantified using gallic acid as standard, and flavan-3-ols, flavonols and stilbenes using (+)-catechin, myricetin and resveratrol, respectively.

Phenols comparison in wines obtained from grapes of vines under different techniques of breeding

A total of 9 simple phenols were identified in all the wine samples (Table 2) including four hydroxybenzoic acids, three hydroxycinnamic acids, one hydroxyphenyl alcohol and one ester. To better understand the phenolic profiling of the wines, they were divided into autochthonous and allochthonous; the obtained data confirm that the amounts of the phenolic compounds vary considerably in different types of wine, depending on the grape variety and these results are in agreement with all the available literature. All wines shared a few common characteristic: the presence of high concentrations of gallic acid that in red wines would be expected since this phenolic acid is principally formed by hydrolysis of flavonoid gallate esters, which are largely absent in white wines, due to the lack of skin extraction (FRANKEL *et al.*, 1995). The data regarding the concentration of individual phenolic acids demonstrated that, excepted gallic acid that

was one of the most important hydroxybenzoic acids in wines, vanillic acid was the highest in Nero d'Avola, Frappato, Nerello Mascalese and Perricone wines (autochthonous vines) and caffeic acid in those from Merlot, Petit Verdot, Cabernet Sauvignon and Cabernet Franc (allochthonous vines), as expected from previous studies (DUGO *et al.*, 2006).

The first objective of the present paper was to investigate the techniques of breeding impact on the phenolic profile of the wines obtained. As seen in Table 2, all the wines obtained from vines bred cortina (CRT) had the highest level of gallic, vanillic and ferulic acid. All the samples had high levels of tyrosol and compared with the wines from the vines bred alberello (ALB) and controspalliera (CSP), the wines from vines CRT contained higher content of this compound. Conversely, all the wines from vines bred CSP contained lower content of tyrosol and gallic and vanillic acids. All the wines had high content of ethyl gallate but it showed the highest level only in three wines from vines bred CRT (Merlot, Frappato and Nero d'Avola). Particularly, differences were not so evident for Nero d'Avola wines while for Cabernet Sauvignon wines the ALB sample showed a considerable difference with the other ones, especially the wine from vines bred CSP.

With regards to the total content of simple phenols quantified using gallic acid as standard, all the wines obtained from vines bred CRT presented the most important concentration; reversely, the wines from vines bred CSP showed the lowest simple phenols compounds concentration in all the samples. From this point of view, the phenolic content of all the wines from vines bred ALB can be considered as intermediate in comparison with the other two groups of wines. Even if the differences among the three groups of samples (ALB, CRT and CSP) were not emphatic, to understand this effect, we considered that the different techniques of breeding could affect the flowering and on this degree exert their related impact on phenolic biosynthesis.

In the present study four flavan-3-ols were observed in all the wine samples (Table 2), including two monomers ((+)-catechin and (-)-epicatechin) and two dimers (procyanidins B1 and B2). The levels of procyanidin B1 and (+)-catechin were the highest for the wines from vines bred CRT; however, considering the procyanidins B1 content, the differences among the three groups of samples were not so categorical. More significant were the differences for (+)-catechin: the highest content was obtained in all the wine samples from vines bred CRT. The content of procyanidn B2 varied among the different samples but generally, with the exception of the Merlot wines, it had the lowest level in the wines from vines bred ALB. About (-)-epicatechin, its content varied remarkably in Cabernet Sauvignon wines so that the sample obtained from vines bred CRT had the highest level of (-)-epicatechin, which was almost 2 times that in the wines from vines bred ALB. However, though (-)-epicatechin presented the highest level in CRT Merlot wine also, its content in wine samples from autochthonous grapes showed different trend, even if the differences among the three groups of samples (ALB, CRT and CSP) were not emphatic. As Table 2 shows, (-)-epicatechin was higher in ALB Nero d'Avola wine and in the CSP Frappato wine.

In spite of the differences evidenced among all the wine samples for each flavan-3-ol detected, considering the total flavan-3-ols content, quantified using (+)-catechin as standard, we can observe (Table 2) that the wine samples with the highest content were those one from vines bred CRT and this trend was evident for wines obtained from allochthonous grapes (Merlot and Cabernet Sauvignon). Some differences in the contents of the individual flavan-3-ols compounds, observed among these regional wines, depend largely on the genotypes of the grape cultivar (TIAN *et al.*, 2008); moreover, it is well known that the accumulation of phenolic compounds in grape berries is strongly affected by terroir factor (GONZALEZ-SAN *et al.*, 1990; RASTIJA *et al.*, 2009). Furthermore, it is well known that leucoanthocyanidin is one common precursor for the synthesis of catechins, epicatechins, and cyanidins, while cyanidins can synthesize epicatechins and cyanidins derivatives. Therefore, a competitive mechanism inevitably exists between catechins and epicatechins or better epicatechins and cyanidins derivatives (CASTELLARIN *et al.*, 2007). However, since the wine-growing region for each grapevine cultivar was geographically the same, the obtained data for total flavan-3-ols content could suggest that the different techniques of breeding could regulate the gene expression related to the flavan-3-ols metabolism, especially for the allochthonous grapes. Therefore, the vines bred cortina (CRT) provide specific characteristics for light exposition or temperature differences between daytime and nighttime that might stimulate the flavan-3-ols metabolism, and thus increase their content, especially in Merlot vine.

In all the wines were identified 9 flavonols. As shown in Table 2, the highest level of total flavonols was observed in all the wines from vines bred CSP. In particular, these wines contained the highest content of rutin, myricetin and quercetin. A high content of isoquercitrin was also detected but the data are not homogeneous as those one related to other flavonols identified. So, Merlot (ALB), Cabernet Sauvignon (CSP), Frappato (CRT) and Nero d'Avola (CSP) wines contained much higher content of isoquercitrin than the other wines, while kaempferol-3-O-glucoside had the highest level in Nero d'Avola and Cabernet Sauvignon (CRT), Frappato (ALB), and

Merlot (CSP) wine samples. Particularly, in Merlot (CSP) and (CRT) wine samples the differences were not so important. Concerning the other individual flavonols, isorhamnetin-3-O-glucoside had the highest level in Frappato, Nero d'Avola and Cabernet Sauvignon (CSP) and in Merlot (CRT). Moreover, Frappato and Merlot (CRT), Nero d'Avola (CSP), and Cabernet Sauvignon (ALB) showed the highest level of kaempferol while higher isorhamnetin content existed in Nero d'Avola and Cabernet Sauvignon (CSP), Frappato (ALB) and Merlot (CRT).

It is difficult to rationalise these results so heterogeneous for many flavonols; rutin, myricetin and quercetin only showed a similar trend. Probably, since several enzymes regulate the flavonoids biosynthesis in grape berries (LI *et al.*, 2011); we can suppose that the cortina technique of breeding could promote the directional flow of carbon to the synthetic branch of rutin, myricetin and quercetin only. As result, these three flavonoids were probably accumulated at a higher level in grape berries, which could explain the high level of these compounds in wines from vines bred CSP. Considering the other flavonoids, the breeding condition might provide grapevine with high activity of a specific enzyme branch pathway in the flavonoid metabolism of grape berries. Consequently, different branch pathway could participate in the regulation of the flavonoid compounds biosynthesis in grape berries, determining the feature of wines.

Stilbenes are one of the minor non-flavonoid phenolic classes of compounds in red wine, with resveratrol being the principal stilbene. Four stilbenes were quantified, two resveratrol geometric isomers and the correspondent glucoside derivatives. As Table 2 shows, the highest level of total stilbenes was observed in all the wines bred CRT and in the Merlot wine the content was particularly high in comparison with the other CRT wine samples; specifically, the total content in CRT Merlot wine was about three times more than that in Frappato and Nero d'Avola CRT wines. The lowest level of total stilbenes was in all the wines from vines bred CSP. The content of the individual stilbenes varied significantly in different types of wine, depending on the grape variety and these results are in agreement with all the available literature. The content of *trans*-resveratrol and the correspondent *trans*-piceid were the highest in all the wines from vines bred CSP; different were the results for *cis*-resvsratrol and *cis*-piceid. Their data were discordant and varied from a sample to another. With regard to Merlot wine samples, *cis*-piceid had levels comparable in ALB and CSP samples, similarly *cis*-resveratrol had the same content in Nero d'Avola in CSP and CRT samples.

From the results discussed above, it is evident a positive effect of the controspalliera technique of breeding that is reflected in an increase in the levels of *trans*-resveratrol and *trans*-piceid. No proportionality between the levels of free isomers of resveratrol and their glucosides is reported (WATERHOUSE and LAMUELA-RAVENTÒS, 1994). As resveratrol, is a naturally occurring phytoalexin produced by some spermatophytes, such as grapevines, in response to injury (BAVARESCO *et al.*, 1999), probably the wines from vines bred CSP presented the highest level of these two compounds because CSP characteristics promoted much high activity of spermatophytes. Thus, the accumulation of *trans*-resveratrol and *trans*-piceid is enhanced. Regarding *cis*-resveratrol and the correspondent *cis*-piceid, it is difficult to rationalise the obtained data, because heterogeneous for many wine samples. However, considering that the synthesis of some phenolics, such as stilbenes, in plants can be induced not only by microbial infections, but also by UV radiation, (BAVARESCO *et al.*, 1999), we suppose that the different techniques of breeding could be correlated with heterogeneous environmental and microclimatic conditions. Therefore, the vine status are directly related to the levels of these phenols and consequently to the phenols biosynthesis in grapes.

Phenols comparison in wines obtained from grapes of vines under two different horticultural cropping methods

The productions for the winemaking cannot ignore the cultivation methods of the vine. It has to be paid particular regard to the planting of vines taking care of the necessary distance from plant to plant and from one row to another so that both guarantees exposition to the sun necessary to the ripening of the grapes. To facilitate exposure to sunlight as well as the collection of fruits, during the winter months the plants are subject to a peculiar technique of pruning the old branches, and no clusters are thinned, while the fruiting branches are bent and attached to proper supports.

The thinning of the branches can only be performed manually and can be execute as follows: 1) after fruit thinning (or setting), 2) at the closing of clusters or 3) at the grape's changing colour (veraison), comparing with a sample that has not been thinned.

Early thinning accounts for less in terms of quantitative reduction, while the best quality results are obtained operating near the veraison. In addition, intervening near the grape's changing colour it is possible to prevent that the physiological adaptation of the growth results in an excessive amount of the berries that could cause pest problems.

This study was conducted to assess the thinning of the branches effect on the phenolic composition of wines from grapes of vines that were subjected to this treatment near the veraison (DRD). For each sample the obtained data were

compare with those one of a sample that has not been thinned (LED).

The results from the analysis of 10 wines are presented in Table 3; in all the DRD samples phenols generally resulted higher than in the control (LED). However, there were varietal differences. As for the other wines considered before, in both wine varieties, gallic acid was the major hydroxybenzoic acid and in Merlot wine the values were higher than in those reported for the other red wines tested. The variation between DRD and LED was marked in Petit Verdot and Cabernet Franc wines, but it resulted scarcely outlined in Perricone wines. The hydroxycinnamic acids profile was in good agreement with the general trend and normally in the DRD samples their content resulted higher than in the control (LED). Caffeic acid was predominant in all the wines from allochthonous grapes. The data concerning hydroxybenzoic and cynnamic acids were distinctive for protocatechuic and caffeic acids in Petit Verdot wine in which the general trend was reversed, even if the differences between the DRD and LED samples were not so significant. These results suggest that other factors besides viticultural treatment can have a significant influence on grape development in this variety. In spite of the differences evidenced, considering the total simple phenol profile quantified using gallic acid as standard, we can observe (Table 3) that the wine samples with the highest content were those one from vine's grapes that were subjected to thinning of the branches near the veraison (DRD).

The amount of (+)-catechin, (-)-epicatechin and procyanidin B1 and B2 were the highest in all the wines from vine's grapes that were subjected to thinning of the branches near the veraison (DRD), confirming that this treatment in-

Table 3 - Occurrence of phenolic compounds in Sicilian wines from grapes of *Vitis vinifera* var. Perricone, Nerello Mascalese, Petit Verdot, Cabernet Franc e Merlot kept under two different horticultural cropping methods (with pruning (DRD) and without pruning (LED) of bunch of grapes). Values are expressed as mean values (mg/L) ± 95% confidence interval (n=3).

	Perricone		Nerello Mascalese		Petit Verdot		Cabernet Franc		Merlot	
Compound	**DRD**	**LED**	**DRD**	**LED**	**DRD**	**LED**	**DRD**	**LED**	**DRD**	**LED**
Gallic Acid	66.34±2.14	63.96±2.14	87.60±2.83	76.67±2.57	81.13±2.62	66.39±2.72	83.71±2.70	64.75±2.17	107.50±3.47	98.57±3.18
Protocatechuic Acid	2.59±0.11	2.16±0.10	5.27±0.22	4.46±0.20	1.86±0.08	1.96±0.08	3.76±0.16	2.06±0.09	9.09±0.38	6.83±0.31
Tyrosol	44.06±3.83	39.00±3.20	43.43±3.77	36.72±3.01	61.82±5.37	51.92±5.06	39.62±3.44	29.00±2.38	88.66±7.70	82.31±6.74
Vanillic Acid	7.69±0.67	6.74±0.62	5.45±0.47	4.31±0.40	6.06±0.53	3.98±0.56	4.62±0.40	3.58±0.33	5.23±0.45	4.60±0.42
Syringic Acid	4.22±0.38	3.57±0.30	1.91±0.17	1.54±0.13	4.51±0.40	2.36±0.38	4.40±0.39	2.48±0.21	2.83±0.25	2.02±0.17
Caffeic Acid	2.95±0.22	2.02±0.15	2.59±0.19	1.82±0.13	9.47±0.71	9.96±0.68	9.48±0.71	5.89±0.42	9.23±0.69	7.15±0.51
Ferulic Acid	0.88±0.03	0.74±0.03	0.73±0.03	0.40±0.02	0.74±0.03	0.73±0.03	1.13±0.04	1.05±0.04	0.86±0.03	0.58±0.02
p-Coumaric Acid	0.38±0.02	0.34±0.02	0.42±0.02	0.32±0.02	1.30±0.07	1.15±0.07	0.62±0.03	0.49±0.03	0.96±0.05	0.92±0.05
Procyanidin B 1	64.69±2.89	63.19±2.67	37.40±1.67	31.67±1.34	44.60±1.99	31.44±1.88	51.85±2.32	34.93±1.47	78.65±3.51	59.10±2.49
Procyanidin B 2	36.94±1.38	25.03±0.93	14.64±0.55	11.59±0.43	29.21±1.09	19.37±1.09	24.18±0.90	15.38±0.57	45.78±1.70	21.25±0.79
(+)-Catechin	105.65±2.36	99.21±2.46	62.76±1.40	51.59±1.28	55.70±1.24	48.83±1.38	61.96±1.38	50.15±1.24	68.04±1.52	45.00±1.12
(-)-Epicatechin	60.14±1.05	56.64±1.10	33.77±0.59	26.75±0.52	60.92±1.06	83.94±1.18	80.01±1.39	75.56±1.46	73.06±1.27	65.39±1.27
Ethyl gallate	13.81±0.38	12.87±0.32	20.18±0.55	16.06±0.40	9.11±0.25	9.02±0.23	13.02±0.36	6.26±0.16	11.23±0.31	7.41±0.18
Rutin	17.63±0.92	17.44±0.87	12.95±0.68	9.94±0.49	13.88±0.72	12.65±0.69	5.98±0.31	3.07±0.15	8.79±0.46	5.35±0.27
Isoquercitrin	33.28±0.74	32.01±0.79	18.89±0.42	15.79±0.39	7.05±0.16	11.06±0.17	6.88±0.15	3.62±0.09	4.35±0.10	5.99±0.12
Isorhamnetin-3-O-glucoside	2.24±0.06	2.15±0.05	3.56±0.09	1.36±0.03	3.59±0.09	2.39±0.09	0.28±0.01	2.67±0.07	1.35±0.03	0.68±0.02
Kaempferol-3-O-glucoside	6.27±0.14	5.78±0.14	1.47±0.03	1.40±0.03	2.66±0.06	3.30±0.07	0.83±0.02	2.13±0.05	2.93±0.07	1.36±0.03
Myricetin	5.78±0.11	5.66±0.14	3.86±0.08	2.56±0.06	19.68±0.39	17.00±0.49	4.46±0.09	4.22±0.10	17.15±0.34	15.08±0.34
Quercetin	14.40±0.50	14.16±0.53	15.69±0.55	13.25±0.49	5.10±0.18	5.33±0.19	1.74±0.06	1.72±0.06	15.22±0.53	14.11±0.53
Kaempferol	0.53±0.03	0.51±0.03	0.53±0.03	0.49±0.02	0.15±0.01	0.18±0.01	0.15±0.01	0.05±0.00	0.15±0.01	0.14±0.01
Isorhamnetin	0.23±0.01	0.22±0.01	0.21±0.01	0.19±0.01	0.20±0.01	0.25±0.01	0.25±0.01	0.07±0.00	0.55±0.02	0.40±0.02
trans-Resveratrol	3.08±0.15	2.94±0.15	0.54±0.03	0.43±0.02	2.08±0.09	1.88±0.09	2.02±0.10	1.65±0.08	6.63±0.33	3.37±0.17
cis-Resveratrol	0.81±0.10	0.68±0.08	0.14±0.02	0.12±0.01	0.79±0.10	0.52±0.10	0.32±0.04	0.18±0.02	3.12±0.39	2.20±0.27
trans-Piceid	4.59±0.28	4.13±0.25	1.74±0.11	1.66±0.10	1.32±0.08	1.16±0.08	2.37±0.15	1.78±0.11	1.99±0.12	1.65±0.10
cis-Piceid	3.51±0.30	3.44±0.27	0.88±0.07	0.83±0.07	0.75±0.05	0.63±0.05	1.02±0.09	0.68±0.05	1.75±0.15	1.45±0.12
Total content										
Simple phenols (mg/L gallic acid)	150.69	138.22	174.92	148.62	188.30	157.67	166.88	120.87	254.58	228.46
Flavan-3-ols (mg/L catechin)	216.78	200.34	122.64	100.01	153.65	158.26	180.12	150.96	203.53	150.70
Flavonols (mg/L myricetin)	59.70	58.32	41.26	38.18	45.92	45.53	16.74	14.93	56.88	50.34
Stilbenes (mg/L resveratrol)	8.63	8.05	2.21	2.01	4.08	3.45	4.32	3.27	11.93	7.38

creases the level of these phenolic compounds into the grape and than their extraction into the wine. (+)-Catechin was the major flavan-3-ol in all the wines from autochthonous grapes and it was predominant in Perricone wine (DRD). The distinctive flavan-3-ol of the allochthonous wines was (-)-epicatechin and only in Merlot wine (DRD) its content was close to procyanidin B1 level that appeared slightly higher. (-)-Epicatechin was distinctive in Petit Verdot wine since its content was predominant in LED wine sample, once again. Higher concentrations of procyanidin B1 and B2 were noticed for Merlot (DRD) wine sample. It is interesting to evidence that their concentrations in DRD samples were about two times higher than in the correspondent control wine samples (LED). The variation in results suggest that other factors besides viticultural treatment can have a significant influence on grape development and regulate the gene expression related to the flavan-3-ols metabolism.

The highest content of total flavonols (estimated using myricetin as standard) was reached in Perricone (DRD) wine sample even if the difference with the (LED) sample was tenuous and this trend was a common denominator among all the wine tested. Looking at the content of each flavonol it emerges that the wines contained appreciable values of rutin, isoquercitrin, myricetin and quercetin; however, their contents were not substantially different between DRD and control wine sample (LED) and were not constantly higher in the DRD samples. In fact, for example, the maximum level of isoquercitrin was reached in Petit Verdot and Merlot wines (LED). This indicate that the flavonol content suffer significant variation; probably it was due to the hydrolysis of flavonol glycosides that were more unstable than aglycon flavonols. Moreover as observed before for the other wine samples, several enzymes regulate the flavonoids biosynthesis in grape berries (LI *et al.*, 2011), consequently, different branch pathway could participate in the regulation of the flavonoid compounds biosynthesis in grape berries, determining the feature of wines.

The stilbenes profile was in good agreement with what observed so far, so the highest level of total stilbenes was observed in all the DRD wine samples. The amounts of the four tested stilbenes varied considerably in the different types of wine, depending on the grape variety and confirming the literature data (DUGO *et al.*, 2006; ROMERO-PÉREZ *et al.*, 1996). The content of *trans*-resveratrol was about two times higher in Merlot wine sample (DRD) than in the correspondent LED one; moreover, in this wine trans and *cis*-resveratrol showed the highest level. Perricone (DRD) wine sample had the highest level of trans and *cis*-piceid while the wine Petit Verdot (LED) presented the lowest level. Of the wine tested, only in Merlot wine samples it was appreciable a difference for *trans*-resveratrol between the DRD and LED treatment. For the other stilbenes this variation between the wine sample from grapes of vines in which the thinning of the branches was performed (DRD), and the sample that has not been thinned (LED) was not so categorical. All of the results presented may be explained, because *trans*-resveratrol and other stilbenes are produced by grapes in response to mould infections and physiological stresses, and their levels in grapes and wines may be different if those phenomena do not appear or appear with different intensity.

Differentiation between autochthonous and allochthonous wines

An attempt to differentiate the autochthonous samples from the allochthonous one was carried out by using the Principal Component Analysis (PCA) and Canonical Discriminant Analysis (CDA) statistical methods. In the present work, the significance of differences in phenolic compound contents between wines obtained from autochthonous and allochthonous vines were estimated by Mann-Whitney U test. The results showed *p*-level below 0.05 for 17 phenols (gallic acid, tyrosol, caffeic acid, ferulic acid, *p*-coumaric acid, (-)-epicatechin, rutin, isoquercitrin, kaempferol-3-O-glucoside, myricetin, kaempferol, isorhamnetin, *trans*-resveratrol, *cis*-resveratrol, total phenols, total catechins, and total stilbenes) which suggested a difference between the two group samples. A Factor Analysis by Principal Components extraction was conducted in order to find a possible discrimination, considering only these 17 variables, which were previously normalized.

Before to perform the Principal Components Analysis, the suitability of the data for factor analysis was checked. The Kaiser-Meyer-Olkin Measure of Sampling Adequacy revealed a value of 0.670, greater than recommended value of 0.500. The Bartlett's Test of Sphericity showed approximate chi-square value equal to 571.039, with a statistical significance at *p*-values < 0.001. Both suggest that the correlation matrix is factored and appropriate for PCA. Thus, the analysis of this matrix showed that the 70% of coefficients presented values with significance higher than 95%. Therefore, all variables have a significant correlation with at least another variable. The highest positive correlations were observed for total phenols/gallic acid (0.962), total stilbenes/*trans*-resveratrol (0.957), total phenols/tyrosol (0.925), *cis*-resveratrol/tyrosol (0.907), myricetin/*p*-cumaric acid (0.896), *cis*-resveratrol/*trans*-resveratrol (0.888), total catechins/caffeic acid (0.867) and total stilbenes/*trans*-resveratrol (0.853), while negative correlations were observed for isoquercetin/caffeic acid (-0.653) and isoquercetin/*p*-cumaric acid (-0.633).

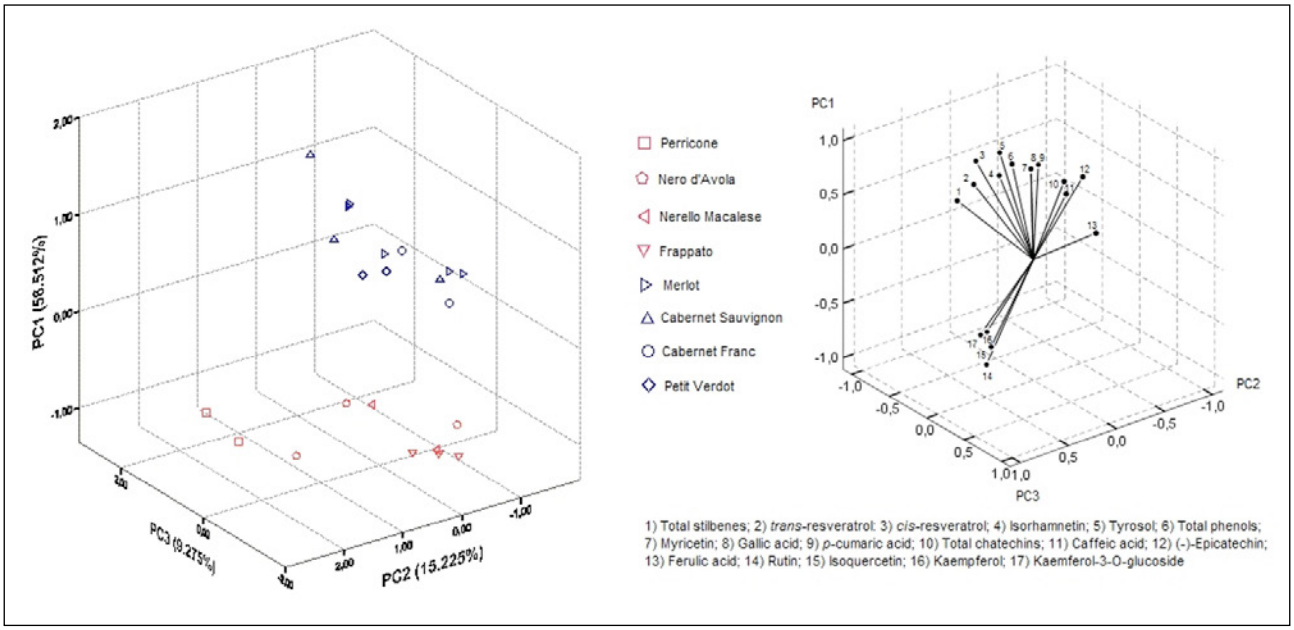


Fig. 1 - 3D Scatterplot of factor scores categorized by typologies. Insert: loading plot for PC1, PC2 and PC3 obtained by Principal Components extraction.

According to Kaiser Criterion, four principal components with eigenvalues exceeding one (9.607, 2.588, 1.577 and 1.190), which explained, respectively, the values of 56.512, 15.225, 9.275 and 7.001% of total variance, were extracted. These components were able to well reproduce all the variables because variables with low saturation in each factor were not identified and communality was always $h^2 \geq 0.672$. The 3D Scatterplot for the 22 wine samples showed two clusters clearly distinguished according to autochthonous or allochthonous variety of vines (Fig. 1). All samples from autochthonous Sicilian grapes showed negative PC1; thus they were characterized by four flavonols, which had the largest negative coefficients in PC1 (rutin, isoquercetin, kaempferol-3-O-glucoside and kaempferol), while total phenols, tyrosol, gallic acid, myricetin, *cis*-resveratrol and total catechins were the variables with the greatest positive association with PC1 and they are characteristic compounds in wines obtained from allochthonous Sicilian grapes.

Only for wine samples obtained from autochthonous vines, the results of Kruskal Wallis test indicated levels significantly different (with $p < 0.05$) for 4 phenolic compounds (gallic acid, *cis*-resveratrol, B1 and B2 procyanidins) in Nero d'Avola, Frappato, Nerello Mascalese and Perricone varieties. The relative correlation matrix was suitable for PCA (KMO value of 0.541 and approximate chi-square value equal to 18.777, with a statistical significance equal to 0.005) and shows that the 50% of coefficients values had significance higher than 95%. The highest positive correlations were observed for procyanidin B1/procyanidin B2 (0.870), procyanidin B2/*cis*-resveratrol (0.728) and procyanidin B1/*cis*-resveratrol (0.640), while important negative correlations were not observed. Two PCs were extracted with eigenvalues exceeding one (2.561 and 1.013), accounting for 89.370% of the cumulative variance. The contributions for PC1 and PC2 were 64.034 and 25.336%, respectively. Variables with low saturation in each factor were not identified and communalities were always greater than 0.750. Procyanidin B1, B2 and *cis*-resveratrol had strongly positive correlation with the first component, while gallic acid was the dominant variable in the second component. A typology separation was obtained in the score plot of PC2 against PC1 (Fig. 2A). Observing the loading factors, gallic acid was more abundant in wines obtained from Nerello Mascalese, while *cis*-resveratrol, procyanidin B1 and B2 in those obtained from Perricone grapes. Wines obtained from Frappato and Nero d'Avola varieties showed the lower contents of gallic acid among all autochthonous, but the wines obtained from Nero d'Avola were characterized by higher content of procyanidin B1 and B2.

Also, the data set constituted by phenolic compound contents of wines obtained from allochthonous vines (Merlot, Petit Verdot, Cabernet Sauvignon and Cabernet Franc) was subjected to Kruskal Wallis test to study the significance of differences, and, in addition, was subjected to PCA in order to check the separation among samples different for the *vitis vinifera* variety. For these data, variables associated with a significant *p*-level (<0.05) were gallic and protocatechuic acids, tyrosol, *cis* and *trans*-resveratrol, *cis* and *trans*-piceid, total phenols, flavonoids and stilbenes. When PCA was applied to the au-

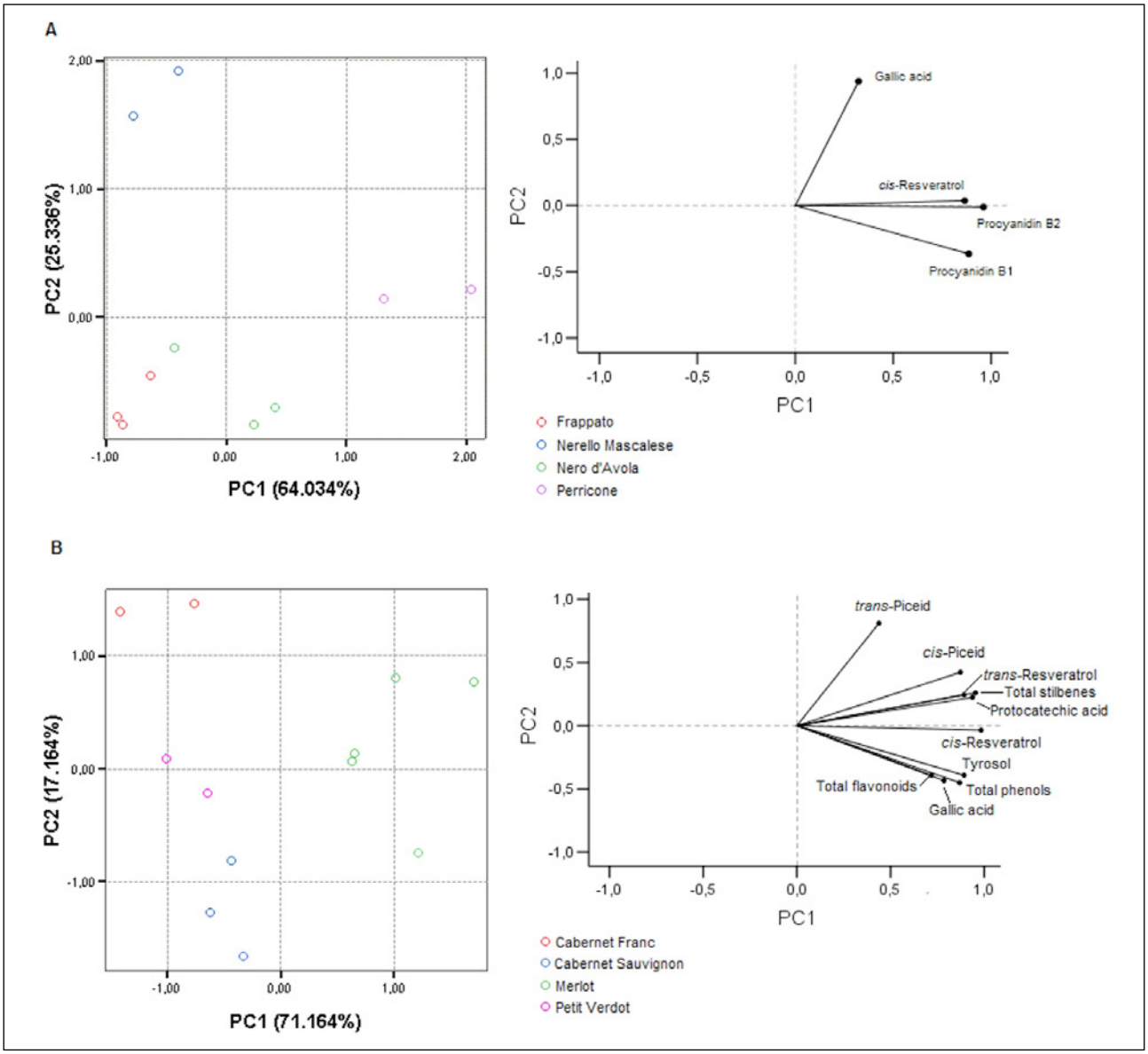


Fig. 2 - 2D Scatterplots of factor scores categorized by varieties of wines obtained from autochthonous (A) and allochthonous (B) vines. Inserts: relative loading plots for PC1 and PC2 obtained by Principal Components extraction.

toscaled data, two principal components with eigenvalues exceeding one (7.186 and 1.716) were extracted according to the Kaiser Criterion. The PCs explain up 89.026% of the total variance (71.862 and 17.164%, respectively). The communalities were always greater than 0.849, except for total flavonoids (0.669). The correlation matrix showed highest positive values for *trans*-resveratrol/total stilbenes (0.969), gallic acid/total phenols (0.952), tyrosol/total phenols (0.949), protocatechuicacid/total stilbenes (0.944), *cis*-resveratrol/total stilbenes (0.939), protocatechuicacid/*cis*-piceid (0.926), and *cis*-piceid/ total stilbenes (0.918). All variables were dominant in the first component excluding *trans*-piceid which had positive correlation with the second component. Fig. 2B shows the PC2/PC1 score plot where can be observed the tendency of groupings for the samples of the same type of wines. PC2 axis split wine samples between Merlot and the others obtained from allochthonous grapes analyzed. The first ones showed always positive PC1 scores while the remaining showed negative PC1 scores. Moreover, samples of Cabernet Franc were separated from the samples of Cabernet Sauvignon by PC1, while samples of Petit Verdot were positioned in an area really near to this axis. Samples of Merlot presented high contents of protocatechuic acid, tyrosol, *trans*-resveratrol, *cis*-resveratrol, *cis*-piceid and total stilbenes. Samples of Cabernet Franc presented high contents of *trans*-piceid and low amounts of tyrosol, *cis*-resveratrol and total phenols. Cabernet Sauvignon wines were characterized by low contents of *cis*- and *trans*-piceid. Whereas, samples of Petit Verdot showed the lowest protocatechuic acid levels compared with all wines obtained from allochthonous variety.

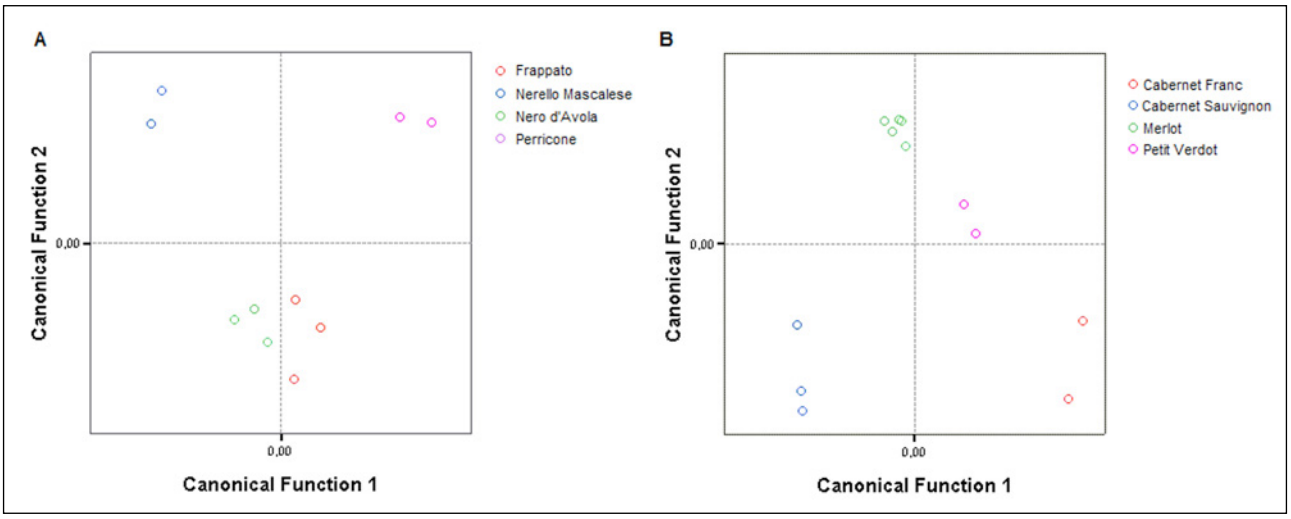


Fig. 3 - Canonical Discriminant Analysis employing grape varieties as grouping criterion for wines obtained from autochthonous (A) and allochthonous (B) vines.

To verify the discrimination of wine samples obtained by PCA, the phenolic compounds, which had significant *p*-values, were employed for CDA, after converting the values into their natural logarithms. About the differentiation between autochthonous and allochthonous samples, the canonical discriminant function obtained using stepwise method showed a high discriminative power, given high correlation value (0.989) and low value of Wilks' lambda (0.022). Thus, it was applied to data set, and each case was assigned to group in which it had achieved the highest value of group membership probability. The classification results indicated that the 100% of total samples were correctly classified. A cross-validation procedure was applied to evaluate the robustness of the classification model. Each sample was in turn omitted from the model estimation, and then its membership was determined from the resulting model. The cross-validation was again 100% correct. Again, similar procedures were applied both for autochthonous and allochthonous data sets. In each case, three canonical discriminant functions, obtained using stepwise methods, contributed to differentiate into classes with high correlation values (0.998, 0.974, and 0.768, 0.990, 0.981 and 0.908, respectively) and low values of Wilks' lambda (0.0001, 0.021, and 0.409, 0.0001, 0.007 and 0.175, respectively). Scatterplots relative to the first two functions for samples obtained from autochthonous (Fig. 3A) and allochthonous (Fig. 3B) vines showed the separation degree among different wine varieties. In fact, the classification results had always indicated that the 100% of samples were correctly classified, with the cross-validation values of 80% and 100%, respectively. Therefore, Canonical Discriminant Analysis had fully confirmed the separation among different wine types.

CONCLUSIONS

The characteristics of the variety itself are important; the compositions in phenolic compounds, observed among these regional wines, indicate that the biosynthesis of these compounds depends not only on the genotypes of the grape cultivar but on the techniques of breeding and cropping methods also.

This preliminary study has provided detailed information on the distribution of 25 bioactive phenolic constituents in selected Sicilian red wines. To the best of our knowledge, for the first time the phenolic composition of red wines from grapes of vines bred alberello was compared with those one of other red wines obtained from grapes of vine breed according more conventional and diffused techniques of breeding (cortina and controspalliera). The results demonstrated substantial differences among the three breeding techniques: the CRT samples had the highest phenolic contents and the CSP ones the lowest; conversely, the flavonols reached the highest values in the CSP samples. The DRD samples had higher phenolic contents than the LED ones. All the different cultivars could be differentiated by PCA and CDA using the phenolic values.

The extension of these works amongst future vintage should refine these conclusions and will constitute additional data to elucidate the phenolic metabolism in grapes.

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PAPER

DISTRIBUTION OF FATTY ACIDS AND TOTAL LIPIDS IN FIVE TISSUES OF EDIBLE SNAIL *HELIX LUCORUM* (L., 1758) FROM THE SOUTHEAST OF TURKEY

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ABSTRACT

This investigation was carried out on fatty acid compositions and total lipid quantities in selected tissues (gonad, mantle, digestive gland, cephalopedal and whole body) of *Helix lucorum* with the aim of evaluating biochemical and nutritional value of edible snail parts. C16:0, C18:0, C18:1ω9, C18:2ω6, C20:2ω6 and C20:4ω6 were found as dominant components. C18:2ω6 (24.44%) and C20:4ω6 (17.49%) were present in fair amounts in the digestive gland and gonad, respectively. The gonad and mantle had a good proportion of C20:2ω6 and the cephalopedal contained significant amount of C20:1ω9. ΣPUFA levels were higher than ΣSFA and ΣMUFA levels in all tissues. Overall ratio of Σω6/Σω3 was defined as follow: gonad, 9.07; mantle, 7.26; cephalopedal, 6.02; whole body, 4.86; digestive gland, 4.06. Among the organs, digestive gland and gonad showed the highest proportion of total lipid. Consequently, the study revealed that tissues of the snail are a good source for PUFA (polyunsaturated fatty acids), essential fatty acids C18:2ω6, C20:4ω6 (precursors of eicosanoids) and other omega 6 family fatty acids, however the total lipid contents were not found at the expected amount. In numerous studies, it is stated that snail meat has several advantages including rich minerals, essential amino and fatty acids and high content of PUFA. Their meat is believed to be a factor affecting higher life span and lower cancer rate in some countries. High amount of C18:2ω6, C20:4ω6 and PUFA make them important nutrition due to preventing cardiovascular disease, inflammatory disorders and retina and brain development.

- Keywords: fatty acids, edible land snail, *Helix lucorum*, tissues, total lipid -

INTRODUCTION

Human beings have been consumed snails for thousands of years and recently snails have been used as food for millions of people worldwide, particularly in some European countries (JESS and MARKS, 1995; MURPHY, 2001). France imports edible snails to a certain extent to meet the needs of its market. Turkey is one of the important countries which export snails to France (ÖZOĞUL *et al.*, 2005) and other European countries; Greece, Germany, Italy and Spain (YILDIRIM and KEBAPÇI, 2004). Some commercially important edible snails including *Cryptomphalus aspersus (Helix aspersa), Theba pisana, Eobania vermiculata, Cantareus apertus, Helix asemnis, Helix cincta* and *Helix lucorum* (YILDIRIM and KEBAPÇI, 2004) are living in Turkish territories.

Nowadays, fatty acids gain more and more importance because of the nutritional and health aspects. Especially, the consumption of marine products rich in omega 3 fatty acids such as C20:5ω3 and C22:6ω3 has increased due to researches demonstrating the beneficial effect of these fatty acids in preventing diseases including cardiovascular disease, inflammatory disorders, retina and brain development (MILETIC *et al.*, 1991; ÖZOĞUL *et al.*, 2005; MILINSK *et al.*, 2006). Snail meat is found to be a factor affecting higher life span and lower cancer rate in Cretan People compared with those of continental Europe (YILDIRIM and KEBAPÇI, 2004).

In addition to basic information about the cultivation and harvesting of edible snails is necessary, their nutritional composition also should be well illuminated. The protein content of edible snail is well documented (ÇAĞILTAY *et al.*, 2011); however there is only limited information on their tissues' lipids and fatty acids composition. The purpose of the present study was to provide quantitative and qualitative information about the lipid and fatty acid composition of some important tissues of *H. lucorum* from the Southeast of Turkey.

MATERIALS AND METHODS

Sampling

Helix lucorum snails were collected in April 2012 from the region of Hevsel Gardens located in Diyarbakır (Altitude: 583 m, Coordinate: N 37° 55.2' / E 40° 13.8'), Turkey. Individually, twelve snails (three for tissues, three for whole body, six for total lipid) similar size (length: 5 ± 0.50 cm, wet flesh weight: 14 ± 0.80 g) were sampled for each tissue lipid analysis. The snails' shells were removed and divided into five groups and their organs (gonad, mantle, digestive gland, cephalopedal and whole body) were dissected out. Then, tissues of each experimental set were conditioned in polyethylene bags and kept at -80°C until chemical analysis.

Chemical analysis

Dissected tissues such as gonad, mantle, digestive gland, cephalopedal and whole body were analyzed in triplicate for proximate composition: lipid content of the snails by the BLIGH and DYER (1959) method. Autoxidation of unsaturated fatty acids was minimized by adding 50 µL of 2% butylated hydroxytoluene in chloroform to each sample during the extraction process. N_2 was used for drying process of lipid extracts. Total lipids were put into reaction vials and the fatty acids were transmethylated by refluxing the fractions in acidified (sulfuric acid) methanol for 90 minute at 85°C. FAMEs (fatty acid methyl esters) of the tissues were extracted from the reaction vials three times with hexane and concentrated (STANLEY-SAMUELSON and DADD, 1983). The total lipid quantities of the tissues were performed according to the method of FOLCH *et al.* (1957).

FAMEs were analyzed by capillary gas chromatography (GC) using Hewlett Packard (Wilmington, DE) gas chromatograph (model 6890), a DB-23 capillary column (60 m × 0.25 mm i.d. × 0.250 µm film thickness and Bonded 50% cyanopropyl) (J. & W. Scientific, Folsom, CA), a flame ionization detector, and Hewlett-Packard ChemStation software. The injection port and the detector temperatures were 270° and 280°C, respectively. The split ratio was 1:20. The flow rates of compressed air and hydrogen were 300 mL/min and 30 mL/min, respectively. Carrier gas was helium (2.8 mL/min). The oven temperature was programmed at a rate of 6.5°C/min from 130°C (1 min hold) to 170°C, then increased at a rate of 2.75°C/min to a 215°C, then again increased at a rate of 40°C/min to 230°C, was held for 12 min. Each tissue fatty acids percentages and spectra of FAMEs are obtained by HP 3365 ChemStation computer program. FAMEs existence and retention times were determined by comparing the spectra of authentic standards (Sigma-Aldrich Chemicals). Individual FAME was identified by comparisons with the chromatographic behaviors of authentic standards.

Statistical analyses

The results were expressed as mean values ± S.D. (Standard Deviation). All analytical determinations were performed in triplicate and the mean values were reported. The analyses were performed using a commercial statistical program (SPSS 20). The percentages of fatty acid were compared by Anova variance analysis with 5% significance level. Tukey's test was used for cooperation of average values.

RESULTS AND DISCUSSION

Nutrient content of edible and nonedible snails was studied up to some extent. Compared with information on fatty acid composition, lipid data correlated with physiological and environmental factors in the snails is notably limited in the literature. Some of the commercially important edible and wild snails including *H. aspersa* (ÇAĞILTAY *et al.*, 2011), *H. aspersa maxima* (MILINSK *et al.*, 2006), *H. pomatia* (ÖZOĞUL *et al.*, 2005), *E. vermiculata* (STAVRAKAKIS *et al.*, 1989), *Helix sp., Haplotrema sportella, Vespericola columbiana* (ZHU *et al.*, 1994) and land slugs *Arion ater, Limax maximus, Prophysaon andersoni (*ZHU *et al.*, 1994) have been studied in terms of fatty acid contents. Almost all data included in molluscan lipid study concern the entire organism and only few reports on the tissue fatty acids are available (RAKSHIT *et al.*, 1997). *Telescopium telescopium* (RAKSHIT *et al.*, 1997), *Bellamya bengalensis*, *Pila globosa* (MISRA *et al.*, 2002), *Unio elongatulus* (EKİN and BAŞHAN, 2010), *Macoma balthica* (WENNE and POLAK, 1989), *Argopecten purpuratus* (CAERS *et al.*, 1999) are some of the tissue studied mollusc species. Although *H. lucorum*, *H. cincta*, *H. asemnis*, *T. pisana*, *E. vermiculata*, *C. asperses* and *C. apertus* are mostly distributing edible snails of Turkey (YILDIRIM and KEBAPÇI, 2004), there is not enough study on their total lipid and fatty acid composition.

C16:0 (6.7-10.1%), C18:0 (9.1-10.7%), C18:1ω9 (12.1-15.7%), C18:2ω6 (11.6-15.2%), C20:4ω6 (13.5-14.3%) were the major fatty acids in *P. andersoni, A. ater, L. maximus* slugs and C16:0 (7.1-9.6%), C18:0 (8.9-10.6%), C18:1ω9 (10.0-12.2%), C18:2ω6 (11.0-15.7%), C20:2ω6 (9.1-12.1%), C20:4ω6 (13.8-16.9%) were major fatty acid in *Helix sp. H. sportella, V. columbiana* snails (ZHU *et al.*, 1994). The high percentages of these fatty acids also found in some other fresh water snails *Theodoxus jordani, Pyrigula barroisi, Melanopsis praemorsum, Melanoides tuberculata,* (GO *et al.*, 2002), *M. praemorsa* (EKİN *et al.*, 2011), in edible land snails *H. aspersa* (ÇAĞILTAY *et al.*, 2011), *H. pomatia* (ÖZOĞUL *et al.*, 2004) and in nonedible snail *T. telescopium* (RAKSHIT *et al.*, 1997). In comparison with above studies, the major fatty acid profiles of *H. lucorum* shown similarities. C16:0 (8.95-14.04%), C18:0 (8.39-11.65%), C18:1ω9 (11.23-18.20%), C18:2ω6 (16.21-24.44%), C20:2ω6 (4.78-10.52%) and C20:4ω6 (8.89-17.49%) were found to be predominant component in all tissues (Table 1). The highest value of C20:4ω6 (17.49%) and C18:2ω6 (24.44%) were detected in the gonad and digestive gland, respectively (Table 2). C18:1ω9 acid was found in high amount in whole body (18.20%) and in the digestive gland (17.18%) (Table 2). However C20:2ω6 was detected in high percentage in the gonad (10.52%) and in the mantle (10.09%) (Table 2). The mostly important essential fatty acid, C18:2ω6 was found in high concentration in the digestive gland (24.44%) (Table 2). Animals (except some invertebrates) cannot synthesize C18:2ω6 from other components, they are obliged to take it from diets and must consume for proper health. A diet only deficient in C18:2ω6 causes most abnormalities such as poor growth, skin lesions, hair loss, poor wound healing, fatty liver and reproductive failure. In *T. telescopium* snail, C16:0 was 12.9% in the digestive gland, 26.2% in the mantle, 21.1% in the cephalopedal and C16:1ω7 was 11.3% in the digestive gland, 6.1% in the mantle, 4.9% in the cephalopedal. Moreover, there were also some strange results in *T. telescopium* snail, for instance C18:3ω3 was not detected in the digestive gland and mantle, but it was found 10.7% in the cephalopedal tissue. Additionally, C20:4ω6 was 7.6% in the digestive gland; it was very high comparing to mantle (RAKSHIT *et al.*, 1997). In edible snail *H. aspersa maxima*, C18:2ω6 was rather a lot, between 44.79-51.19% (MILINSK *et al.*, 2006). In contrast, a comparison between the components acids of the cephalopedal, digestive gland and whole body of *H. lucorum* showed some points of interest; C18:3ω3 was at high percentages in the digestive gland; the percentages of C22:0 varied from 2.10% (in the digestive gland) to 4.19% (in the whole body); C12:0, C13:0, C14:0, C15:0, C20:0, C14:1ω7, C17:1ω7 and C22:6ω3 were detected less than 1% in all tissues; C20:1ω9 is present in significant amount (4.47%) in the cephalopedal tissue (Table 1). Snail cephalopedal is considered as a storage organ (JOHNS *et al.*, 1979), probably, the cephalopedal stored this fatty acid for further metabolic activities. C20:1ω9 was also obtained in high percentages in *U. elongatulus* cephalopedal (EKİN and BAŞHAN, 2010).

The Σω6/Σω3 ratio has been suggested to be useful indicator for comparing nutritional values of tissues lipids. The ratio of ω6/ω3 was 9.07 (in the gonad), 7.26 (in the mantle), 6.02 (in the cephalopedal), 4.86 (in the whole body) and 4.06 (in the digestive gland) (Table 1). The percentages of ω6 was 50.27, 45.42, 41.04, 42.81 and 41.54% in the gonad, mantle, digestive gland, cephalopedal and whole body, respectively (Table 1). In addition, *L. maximus, P. andersoni, A. ater* slugs and *H. sp. H. sportella, V. columbiana* (ZHU *et al.*, 1994) species contained high amount of Σω6 than Σω3. However, in the most of the previous studies, it was reported that the percentage of Σω6 was lower than Σω3 in marine molluscs (ABAD *et al.*, 1995; PAZOS *et al.*, 2003). Fish is well known to be the major source of ω3 fatty acids in the diet and people have been aware of the health benefits associated with consuming fish. The useful ω3 in fish flesh are C20:5ω3 and C22:6ω3 (ÖZOĞUL *et al.*, 2005). Unlike fish and marine gastropods, level of ω3 in *H. lucorum* accounted for low quantity. C22:6ω3 level varied from 0.03% (in the gonad) to 0.22% (in the whole body) and level

Table 1 - Fatty acid profiles of selected tissues from edible snail *Helix lucorum*.

Fatty acids	Gonad (Mean•±S.D.)••	Mantle (Mean•±S.D.)•	Digestive gland (Mean•±S.D.)•	Cephalopedal (Mean•±S.D.)••	Whole body (Mean•±S.D.)••
Saturated					
C12:0	0.07±0.01a	0.03±0.01b	0.04±0.01b	-	0.09±0.02a
C13:0	-	0.02±0.01a	0.03±0.01a	0.06±0.01b	0.07±0.01b
C14:0	0.30±0.03a	0.66±0.05b	0.89±0.08c	0.40±0.05a	0.52±0.04d
C15:0	0.19±0.02a	0.31±0.03b	0.36±0.04b	0.25±0.03a	0.72±0.06c
C16:0	10.94±0.86a	14.04±1.19b	13.17±1.13b	8.95±0.81c	12.55±1.07ab
C17:0	1.44±0.13a	1.25±0.11a	2.13±0.19b	2.19±0.20b	0.70±0.07c
C18:0	10.78±0.88a	11.65±1.01a	8.39±0.78b	11.42±1.12a	8.92±0.75b
C20:0	0.14±0.02a	0.24±0.03b	0.22±0.03b	0.19±0.02b	0.16±0.02a
C22:0	3.01±0.18a	3.05±0.22a	2.10±0.16b	3.45±0.25a	4.19±0.32c
ΣSFA	**26.87±1.33a**	**31.25±1.65b**	**27.33±1.43a**	**26.91±1.27a**	**27.92±1.39a**
Monoenoic					
C14:1ω7	0.04±0.01a	0.10±0.02b	0.06±0.01a	-	0.11±0.02b
C16:1ω7	3.82±0.33a	3.72±0.29a	3.53±0.26a	3.22±0.24a	2.98±0.23a
C17:1ω7	0.21±0.03a	0.08±0.01b	0.06±0.01b	0.11±0.02c	0.12±0.02c
C18:1ω9	13.14±1.01a	11.23±0.92b	17.18±1.18c	15.76±1.12ac	18.20±1.27c
C20:1ω9	0.27±0.03a	1.79±0.15b	0.29±0.04a	4.47±0.34c	0.52±0.05d
ΣMUFA	**17.48±1.15a**	**16.92±1.13a**	**21.12±1.20b**	**23.56±1.26c**	**21.93±1.18b**
Polyenoic					
C18:2ω6	16.12±1.17a	18.77±1.35b	24.44±1.56c	19.21±1.43bc	20.78±1.48bc
C18:3ω3	2.83±0.22a	4.34±0.37b	7.30±0.54c	4.99±0.31b	6.41±0.53bc
C20:2ω6	10.52±1.04a	10.09±1.17a	4.78±0.31b	8.28±1.14c	6.55±0.62d
C20:3ω6	4.25±0.37a	3.09±0.22b	1.76±0.15c	1.86±0.16c	2.09±0.17c
C20:4ω6	17.49±1.26a	12.25±1.05b	8.89±0.77c	12.76±1.12b	11.23±1.02b
C20:5ω3	2.68±0.24a	1.88±0.16b	2.70±0.21a	2.03±0.19b	1.91±0.13b
C22:5ω6	1.89±0.19a	1.22±0.09a	1.17±0.16a	0.70±0.08b	0.89±0.10b
C22:6ω3	0.03±0.01a	0.04±0.01a	0.10±0.02b	0.09±0.02b	0.22±0.03c
ω6	**50.27**	**45.42**	**41.04**	**42.81**	**41.54**
ω3	**5.54**	**6.26**	**10.10**	**7.11**	**8.54**
ω6 / ω3	**9.07**	**7.26**	**4.06**	**6.02**	**4.86**
ΣPUFA	**55.81±2.12a**	**51.68±2.03b**	**51.14±2.03b**	**49.92±1.99b**	**50.08±2.08b**

Results expressed as percentage of total fatty acids methyl esters.
*Values are means ± S.D (Standard Deviation) for three samples of triplicate analysis.
**Means followed by different letters in the same line are significantly different ($P < 0.05$) by Tukey's test.
ΣSFA: Saturated Fatty Acids, ΣMUFA: Monounsaturated Fatty Acids, ΣPUFA: Polyunsaturated Fatty Acids, ω6: Total of omega 6 fatty acids, ω3: Total of omega 3 fatty acids.

Table 2 - Major fatty acids compositions (%) of the tissues from *Helix lucorum*.

Tissues	Predominant six fatty acids of *Helix lucorum* and their percentages (%)						Total percentages (%)
Gonad	C20:4ω6 (17.49)	C18:2ω6 (16.12)	C18:1ω9 (13.14)	C16:0 (10.94)	C18:0 (10.78)	C20:2ω6 (10.52)	78.99
Mantle	C18:2ω6 (18.77)	C16:0 (14.04)	C20:4ω6 (12.25)	C18:0 (11.65)	C18:1ω9 (11.23)	C20:2ω6 (10.09)	78.03
Digestive gland	C18:2ω6 (24.44)	C18:1ω9 (17.18)	C16:0 (13.17)	C20:4ω6 (8.89)	C18:0 (8.39)	C18:3ω3 (7.30)	79.37
Cephalopedal	C18:2ω6 (19.21)	C18:1ω9 (15.76)	C20:4ω6 (12.76)	C18:0 (11.42)	C16:0 (8.95)	C20:2ω6 (8.28)	68.10
Whole body	C18:2ω6 (20.78)	C18:1ω9 (18.20)	C16:0 (12.55)	C20:4ω6 (11.23)	C18:0 (8.92)	C20:2ω6 (6.55)	78.23

of C20:5ω3 was between 1.88% (in the mantle) and 2.70% (in the digestive gland) (Table 1). The highest value of ω3 was found to be 10.10% in the digestive gland because of relatively high percentage of C18:3ω3 (7.30%) (Table 1). The differences in the level of ω3 and ω6 fatty acids of species is potentially due to dietary factors because marine plankton are rich in ω3 fatty acids, while terrestrial and freshwater planktons are rich in ω6 acids (SARGENT, 1976). Additionally, endogenous factors such as genetic, physiology, biochemical reaction etc. may

Table 3 - Total lipid content of the tissues from *Helix lucorum* (g/100 g wet tissue ±S.D. % on basis of total lipid).

Tissues	Gonad	Mantle	Digestive gland	Cephalopedal	Whole body
Total lipid	2.52±0.40	0.93±0.14	3.54±0.37	1.21±0.13	1.09±0.23

affect accumulation of ω3 and ω6 fatty acids in the species tissues.

The maximum amount of ΣSFA (saturated fatty acids), ΣMUFA (monounsaturated fatty acids) and ΣPUFA (polyunsaturated fatty acids) were found in the mantle (31.25%), in the cephalopedal (23.56%) and in the gonad (55.81%), respectively. It is of the interest that a considerable amount of ΣPUFA was detected in all tissue analyses. ΣPUFA level of each tissue was higher than 50% (except cephalopedal tissue which is 49.92%) nearly equal to sum of ΣSFA and ΣMUFA levels. ΣPUFA levels of the tissues detected high because of high concentration of C18:2ω6, C20:2ω6 and C20:4ω6 (Table 1). It was reported that ΣPUFA level was higher than ΣMUFA and ΣSFA levels in *L. maximus, P. andersoni* and *A. ater* slugs (ZHU *et al.*, 1994). In edible snail *H. aspersa*, sum of ΣMUFA and ΣPUFA was 55.02% and ΣPUFA was at the highest level (ÇAĞILTAY *et al.*, 2011). In an another edible snail *H. aspersa maxima*, ΣPUFA levels were also obtained at high percentages (between 46.40-54.97%) (MILINSK *et al.*, 2006). On the other hand, it has been expressed that high ΣSFA (37.87%) and low ΣPUFA (25.83%) level were found in edible snail *H. pomatia* (ÖZOĞUL *et al.*, 2005). Briefly, tissues of *H. lucorum* are characterized by high percentages of PUFA. However, lipid and fatty acid content of a snail tissue mostly depends on several factors such as seasonal changes, nutritional availability, environmental factor, physiology, reproduction, tissue functions, etc. The notable difference being the high level of ΣPUFA with a somewhat decreased relative concentration level of ΣSFA and ΣMUFA in the snail tissues probably relative to temperature conditions because, the samples were collected during April when temperature is still low (approximately 15°C).

Prostaglandins are considerably important molecules in mollusc physiology such as possible renal function, ion regulation in gill, and reproductive functions in gonad (STANLEY-SAMUELSON, 1987). It is empathized that egg production in freshwater snail *Helisoma durgi* was stimulated by prostaglandins (KUNIGELIS and SALEUDDIN, 1986). In comparison with other fatty acids, C20:4ω6 (precursors of prostaglandins) was in fair amount in the tissues particularly in the gonad (17.49%). Probably, this high value is related to reproduction activity or accelerated feeding activities after emerging from hibernation.

The digestive gland (3.54%) and gonad (2.52%) of the snail exhibited maximum reserve of total lipid whereas mantle (0.93%) and cephalopedal (1.21%) contained relatively low quantities (Table 3). This signifies that digestive gland and gonad are more active organs. It was declared that these organs had major proportion of total lipid in gastropod (VOOGT, 1983). In the study of *T. telescopium*, total lipid amount was found to be 36.91% in the digestive gland, 13.44% in the mantle, 11.67% in the cephalopedal (RAKSHIT *et al.*, 1997). The results are very high for an edible land snail. The most of the studies mentioned that land snails contain low quantities of lipid (ÖZOĞUL *et al.*, 2005; MURPHY, 2001). Total lipid percentages of *H. aspersa maxima* varied between 0.45% and 2.66% (MILINSK *et al.*, 2006).

In conclusion, given improved cultural techniques, *H. lucorum* become the potential a rich source of unsaturated fatty acids; C18:1ω9, C18:2ω6, C20:2ω6 and C20:4ω6 (ω6 fatty acids) that the snail meet contains significant amount of essential fatty acid (C18:2ω6). PUFAs with more than 20 C atoms are indicating that the use of this food is good for diets, irrespective of total lipid content. In addition, the results can be important guide for further investigation on physiological, biochemical and systematic studies of molluscs.

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PAPER

BERHI DATES PITS-ENRICHED BREAD: EFFECT ON DOUGH RHEOLOGY, BREAD QUALITY, AND SHELF LIFE

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ABSTRACT

Dates Pits Powder (DPP) was added to bread flour intended for formulations contained 4, 8, and 12% DPP. The enriched bread could deliver 1.33, 0.88, and 0.44 g of soluble fiber / per serving which meets FDA requirement for health claim. Higher Farinograph water absorption and mixing tolerance (MTI) was increased due to DPP addition, as well as the water holding capacity. The dough resistance to extension (RE) of the control flour has significantly increased ($p \leq 0.5$) at all DPP contents. Bread loaf volume was significantly dropped whereas the color of the crumb became darker in color. Bread staling increased as a function of DPP levels, but this effect was limited to storage temperature and DPP level rather than storage time. The 0% DPP blend stored at 25°C showed the least firmness, while the greatest firmness recorded was the control stored at room temperature for 5 days.

- Keywords: dates pits, dough, dough rheology, bread staling -

1. INTRODUCTION

Dates palm trees (*Phoenix dactylifera*) are common in the Middle East. The seed constitutes about 10-15% of the fruit and mostly used as animal feed (HUSSEIN *et al.*, 1998). The chemical composition of the date seeds is about 5% moisture, 6% proteins, 11% fat, 1.5% ash, 52% dietary fiber and high level of phenolics (33102-4430 mg/100 g) (ALFARSI *et al.*, 2007; HAMADA *et al.*, 2002). MOHAMED *et al.* (2010) reported the addition of defatted dates seed fiber concentrate to replace wheat flour in bread at 1 and 3%. Doughand bread evaluation revealed that, fine concentrate caused decrease in bread loaf-volume, change in crumb color and slightly increase in bread crumb firmness. On the other hand, the addition of coarse material significantly affected dough mixing characteristics, decreased loaf volume, bread crumb firmness ($P < 0.05$) and increased the fiber content of bread. AL-FARSI (2008) optimized dietary fiber, antioxidants extraction using different organic solvents, and showed that dates pits can be an in-expensive source of dietary fiber and antioxidants. The thermal analysis of defatted date pits revealed no sign for glass transition which means very low molecular mobility or high crystallinity of the carbohydrates content (RAHMAN *et al.*, 2007). The mathematical modeling of water absorption by date pits reached its saturation at 41.5% after 10 days (JI *et al.*, 2004). Compared to oven and sun drying, freeze drying gave the maximum date-fiber concentrate with the highest swelling, water holding and oil capacities (CHEMA *et al.*, 2011).

Many reports in the literature referring to dietary fiber-enrich bread and the effect of fiber on the dough and the final quality of bread (ANIL, 2007; COLLAR *et al.*, 2007; UYSAL *et al.*, 2007; YUTHANA *et al.*, 2008). Scientists have described soluble fiber component as cholesterol lowering agent when consumed in a low fat diet (WU *et al.*,1994). A health claim can be made as allowed by FDA when soluble fiber is consumed at 3 g per day (Food & Drug Administration, 1997) or 0.72 g per serving (one bread serving is 30 g as is or 21 g dry basis). The objectives of the study were (a) to introduce a new local source of soluble fiber by using dates pits, a by-product of the dates industry in Saudi Arabia, the largest producer of dates in the world. (b) increase the amount of soluble fiber in bread by adding the appropriate amount of dates pits powder to bread formulation to a minimum of 0.75 g per serving (2.5% as is or 3.5% dry basis), and (c) to examine the effect of dates pits powder on flour dough rheological properties and over all fresh and stored bread quality.

2. MATERIALS AND METHODS

2.1. Collection and preparation of raw materials

Wheat flour (Qatar Flour Mills Co.) was purchased from a local market. The composition of wheat flour provided by the supplier as, fat 1.3%, total carbohydrates 72.6%, protein 12.5% and crude fiber 0.2%. The Berhi date pits were obtained from the dates processing unit of the Dates Industries and Research chair, King Saud University, Riyadh, Saudi Arabia. Pits were collected the dates processing at three different processing periods which was based on new incoming load of fresh dates. Each pits batch was treated separately and considered a rep. The date pits were washed thoroughly to remove all types of mucilaginous material then dried under shade and stored. The milling of date pits was done in two steps using two different mills to obtain date pits powder (DPP). At first, date pits were coarsely ground using Retsch mill, 5657 HAAN, Germany. The coarsely ground date powder grits were further reduced in size using Perten Laboratory Mill 3100, Perten Instruments Sweden. The powder obtained from Perten mill was then sieved through 60 and 80 mesh sieves and stored separately at 4°C till further use. The baking ingredients like salt, sugar, shortening, yeast, milk powder and improver were also purchased from a local market.

2.2. Preparation of wheat flour and DPP blends

Date pit powder/wheat flour blends were prepared by replacing wheat flour with 60 or 80 mesh DPP at 4, 8 and 12% levels in triplicates. The levels were chosen after a trial for up to 20% replacement, but the dough handling was impossible beyond 12%. This level was also delivered enough fiber to make health claim as mentioned above. Wheat flour without DPP was used as a control in all types of analysis.

2.3. Dough Properties

2.3.1. Farinograph studies

Wheat flour and blends were tested using 50 g mixing bowl on a computerized Farinograph according to the AACCI (2000) approved method No. 54-21. The results were reported for water absorption (%), development time (minutes), dough stability time (minutes), mixing tolerance index (BU), time to break down (minutes) and Farinograph quality number (FQN).

2.3.2. Water holding capacity

Water holding capacity (WHC) of wheat flour/blends was determined according to the method

adopted by TRAYNHAM *et al.* (2007) with some modifications. Wheat flour or blends (1.5 g) were dispersed in 28.5 mL of distilled water in a 50 mL centrifuge tube. Tubes were agitated for 10 min, let to rest for 1 h, and then centrifuged at 4,500 rpm for 30 min. After decanting the supernatant, each tube was weighed and WHC (g of water/g flour) was calculated as:

WHC= [(wt of tube after decanting - wt of dry tube) - total flour wt (g)] / total flour wt (g)

2.3.3. Extensograph testing

Stretching properties of wheat flour and blends were determined using a computerized Brabender extensograph (Brabender, Duisburg, Germany). Flour doughs were prepared in Farinograph, cut into three parts of 150 g each, and passed through the balling unit of the extensograph following the short method with reduced proofing time. After 30 min resting in the fermentation cabinet, the dough was stretched. After this first test, the balling and moulding operations were repeated and the dough was tested after another resting time of 30 min (total 60 min). The same procedure was repeated for a third time after 90 min rest time. The results were expressed as: (a) energy (E), the work applied for stretching the dough and is an index of the flour quality (expressed in cm^2); (b) resistance to constant deformation after 50 mm stretching (R50); (c) extensibility (Ex), is indicated by the length of the curve expressed in millimeters (mm); (d) maximum BU is the maximum height of the curve; (e) R/Ex Ratio indicates the balance between dough strength (resistance to extension) and the extent to which the dough can be stretched before breaking (extensibility).

2.4. Bread properties

2.4.1. Baking procedure

The quantities of dry ingredients were calculated as percent based on flour weight. Baking performance of the control flour and blends was determined on pup loaves using AACCI (2000) Method No. 10-09. The 100 g blends were mixed with 3 g instant dry yeast, 6 g sugar, 0.015 g improver (High Power Improver: wheat flour, antioxidant E 300, alpha amylase, hemicellulase), 4 g fat free instant milk powder, 1.5 g salt and 5 g shortening. The amount of water used for making the dough was in the range of 63% (control) to 66% for various blends as determined by mixing and feeling the dough. The mixing time was 9 and 11 min for the control and blends, respectively. The dough was proofed for 60 min, punched and proofed for another 30 min. After second proofing and punching, the dough was sheeted, moulded, placed in the pan, and proofed for another 30 min prior to baking. All loaves were baked at 220°C for 20 min. The baked breads were allowed to cool at room temperature for two hours before loaf volume was recorded. Breads were then sliced and used for other analysis.

2.4.2. Crumb color

Bread crumbs of the control and the blends were dried at room temperature and powdered in coffee grinder. The color values: L*(Lightness), a* (Redness) and b* (Yellowness) of dried bread crumb powders were determined using SATAKE Color Grader, NCGA, Japan with a D65 light source.

2.4.3. Bread firmness

Bread firmness was determined using a texture analyzer (TA HDI ® Stable Micro system Texture Analyzer) following the AACCI (2000) Method 74-09. Bread slices were stored at three different temperatures: room temperature, 5° and -20°C for 7 days. Firmness using 3 center slices (30 mm thick) was determined on day 2, 5, and 7 using 5 mm cylindrical Probe with 50 kg load cell. The only sample tested after one day of storage is the room temperature while the all samples were tested at all DPP levels and temperatures. The data was processed using Texture Expert Exceed ®software.

2.5. Rapid Visco-Analyzer measurements (RVA)

2.5.1. Pasting properties

Pasting properties of the wheat flour blends were determined using a Rapid Visco Analyzer (Newport Scientific, Sydney, Australia). Wheat flour or blends (3.5 g at 14% moisture basis) were directly weighed into aluminium RVA canisters and distilled water was added to achieve a total weight of 28.5 g. The obtained slurry was held at 50°C for 50 s, heated to 95°C in 3:42 min (at 12.16°C/min) and held at 95°C for 2 hr and 30 min. It was then cooled to 50°C in 2 min (at 11.84°C/min) and held at 50°C for 2 min. The rotating speed of paddle was 960 rpm for the first 10 s and then reduced and kept at 160 rpm throughout the remainder of the experiment. All measurements were done in triplicate and the Thermocline window software was used to process the data.

2.5.2. Gel texture

Gel texture parameters were determined on the gels prepared in the RVA as described above. The gels (35 mm in height) were transferred into 25 mLbeakers having internal diameters of 30 mm and stored overnight at room temperature. Gels were compressed using Brookfield CT3 Tex-

ture Analyzer (Brookfield Engineering Laboratories, Inc. Middleboro, USA) in two penetration cycles at a speed of 0.5 mm/s to a distance of 10 mm using 12.7 mm wide and 35 mm high cylindrical probe. Gel hardness, springiness, cohesiveness and adhesiveness were recorded. The gumminess was calculated as a product of hardness and cohesiveness while chewiness as a product of gumminess and springiness.

2.6. Statistical analysis

All measurements were done in triplicate. One way analysis of variance technique was used to study the effect of DPP levels on wheat flour rheological and baking properties. Duncan's Multiple Range (DMR) test at $p \leq 0.05$ was used to compare means using PASW® Statistics 18 software.

3. RESULTS AND DISCUSSION

3.1. Dates pits fiber contents distribution

The proximate fiber composition of wheat flour (hard red spring wheat) were; 0.66, 0.97, and 1.63% for soluble fiber, insoluble fiber and total dietary, respectively (MOHAMED *et al.*, 2008). The dates pits fiber content was determined for the samples taken from the same Dates Industries and Research chair, King Saud University, Riyadh, Saudi Arabia and tested according to AOAC method 962.09 (AOAC 190) (Huthail Najib and Yousef M. Al-Yousef). The soluble fiber content of dates pits powder (DPP) (60 or 80 mesh) was calculated by subtracting the sum of cellulose (40%), hemicellulose (15%) and lignin (8%) from the total dietary fiber content of DPP (100%) which was turn out to be 37%. The soluble fiber content of the 12% DPP, 8% DPP, and 4% DPP-containing bread samples were 1.33, 0.888, and 0.444 g / bread serving as is (one bread serving is about 30 g), respectively. The term "as is" when it is used for bread, it means that one bread serving is 30 g as is or 21 g dry basis considering the bread moisture content is 30% on the average. Based on these results, the requirements set by the FDA (0.75 g/serving as is or in 21 g dry basis), the 12 and 8% DPP breads can be considered as meeting the FDA requirement for health claim. These calculations were made without including the soluble fiber naturally exists in the bread flour.

3.2. Farinograph

The water absorption of the blends has increased by about 1.5% compared to the control in the presence of both pit powder (60 or 80 mesh). As seen in Table 1, samples containing 12% DPP exhibited lower water absorption than the control, which could be due to the increase in oil content naturally found in DPP (about 11% oil content). The presence of oil could accelerate gluten network formation thus the overall water distribution dynamics in the dough.

Mixing flour and water for dough formation is the most critical step of dough development that requires precise mixing time and exact amount of available water. The significance of dough mixing is evident in the di-sulphide bond formation between gluten (wheat proteins) frac-

Table 1 - Farinograph data of Hard Red Spring wheat flour blended with different levels of dates pits powder of 60 and 80 mesh particle size.

	Wheat flour + Date pit powder 60 mesh			
	0%	**4%**	**8%**	**12%**
Water absorption (%)	63.77±0.15b	64.17±0.21c	64.63±0.06a	63.07±0.31d
Development time (min)	8.43±0.15d	10.23±0.96c	10.90±0.36b	15.80±0.53a
Dough stability time (min)	17.16±1.06b	16.43±0.97b	18.70±0.69a	19.07±0.21a
Mix tolerance index (BU[1])	21.33±4.16c	29.67±6.66bc	34.67±8.08ab	46.00±7.21a
Time to break down (min)	16.80±2.55ab	16.00±0.62b	18.47±2.66ab	20.00±0.00a
FQN[2]	168.00±25.53ab	160.00±6.24b	184.67±26.56ab	200.00±0.00a
	Wheat flour + Date pit powder 80 mesh			
Water absorption (%)	63.77±0.15c	64.27±0.15b	64.57±0.15a	63.20±0.17d
Development time (min)	8.43±0.15b	9.20±0.26b	10.70±0.44b	14.30±2.46a
Dough stability time (min)	17.16±1.06ab	15.07±2.70b	17.80±1.99ab	18.83±0.15a
Mixing tolerance index (BU)	21.33±4.16b	26.67±8.08b	27.00±3.61b	39.33±5.03a
Time to break down (min)	16.80±2.55ab	15.40±2.07b	18.20±2.38ab	20.00±0.00a
FQN	168.00±25.53ab	154.00±20.66b	182.00±23.81ab	200.00±0.00a

Means carrying same letters in rows are statistically non-significant.
[1]BU=Brabender Units;
[2]FQN=Farinograph Quality Number.

tions. Mixing results in gluten film formation, which is essential for enfolding dough components together, thus forming a viscoelastic material (dough). Farinograph testing was employed to determine the effect of DPP on the water absorption and the mechanical properties of the formed dough. The water required for dough maximum consistency in the Farinograph was different from that needed for the dough used for baking. That is due to the presence of additional ingredients to the bread formulation beside the flour/water used for Farinograph in addition to the different type of mixing used relative to the Farinograph mixing (Table 1). Despite the difference in the amount of water required for dough formation, the Farinograph is an appropriate tool to test the effect of DPP on the mechanical rheology of wheat flour. DPP presence has increased the Farinograph water absorption of the control by 3% for the blends. The dough mixing tolerance index (MTI) is defined as the difference in Brabender Units (BU) between the top of the Farinograph-profile peak and 5 min later, where larger differences indicate increasing DPP influence on the control. MTI of wheat flour had increased in the presence of the 60 and 80 mesh DPP, where MIT of the 4, 8, and 12% 60 mesh DPP were increased from 21.3 BU (the control) to 29.7, 34.7, and 46 BU, respectively. The 80 mesh DPP added at 4, 8, and 12% has increased MIT form 21.3 to 26.7, 27.0, and 39.3 BU, respectively (Table 1). Further increase in DPP content resulted in additional increase in MIT of the control but, there was slight difference between 4 and 8% blends. Dough stability has significantly increased at 8 and 12% DPP ($p \leq 0.5$) compared to the control (control 17.2 min and blends 18.7 or 19.1 min) due to high oil content of the DPP. Dough stability is defined as the difference in min between the time when the top of the curve reaches 500 BU and the time where it departs the 500 BU. Once again, particle size of DPP has different effect on the dough stability, where 60 mesh has more influence than the 80 mesh. The effect of DPP on the Farinograph profile of the control could be attributed to the presence of fiber and the relatively high oil content. The influence of these components on MIT and dough stability is due to their ability to either compete with gluten for water (fiber) or help gluten to spread and form the network needed for dough formation (oil) thus influencing the overall dough mechanical rheology. This way, the quality of the final product, such as loaf volume and crumb quality, will be influenced. The arrival time is also one of the dough quality parameters obtained from the Farinograph testing and described as is the time needed for the curve to reach the 500 BU mark, where longer arrival time indicates slower gluten development during mixing. Consequently, this will increase dough mixing time and delay bread production. The arrival time for the control flour was 1 min while the 12% blend needed 2.2 min to arrive. The overall dough development time of the control has significantly increased from 8.5 min to 15.8 min for the 12% blend (Table 1). This increase is attributed to the competition between the flour and DPP components for water as well as the high oil content in DPP. The 80 mesh sample required 14.3 min development time for the 12% blend.

3.3. Water holding capacity

Water-holding capacity (WHC) is an important protein–water interaction that occurs in different types of food systems which was defined as the ability of a protein network to absorb and retain bound, hydrodynamic, capillary, and physically entrapped water (DAMOKARM, 1997). Several wheat flour components are documented to influence water-retention potential, including pentosans, damaged starch, and glutenin. The water holding capacity of the control flour has significantly increased at all DPP blends, where the 8% DPP exhibited the highest value. The 12% blend showed lower value than the 8% possibly due to the high oil content of this blend which contains more oil than the 8%. The particle size difference between 60 and 80 mesh seems to be too narrow to cause significant difference in the water holding capacity of the control flour (Fig 1). The drop in water holding capacity at higher DPP could be due to higher oil content interaction with glutenin which prevented gluten from water absorption.

3.4. Extensograph properties

The data collected from an Extensograph includes a force-time curve for a piece of dough stretched until it breaks. The measured param-

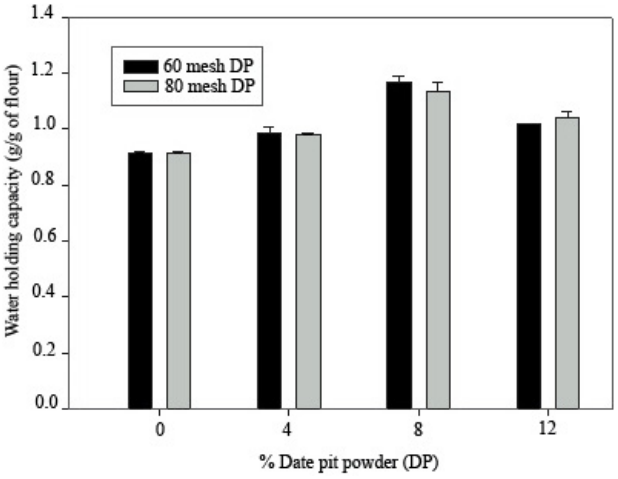


Fig. 1 - Water holding capacity of control wheat flour blends with different levels of date pit powder of 69 and 80 mesh particle size.

Table 2a) - Extensograph data of Hard Red Spring wheat flour blended with different levels of dates pits powder of 60 mesh particle size.

Dough rest time	DP Level	Energy (cm^2)	Resistance to extension (BU)	Extensibility (mm)	Max (BU)	Ratio No.
30 minutes	0%	104.3±4.9a	456.3±42.5b	136.3±5.0a	570.7±57.6a	3.3±0.5c
	4%	94.3±13.8b	458.3±24.8b	129.3±10.1a	534.7±47.1a	3.6±0.1c
	8%	90.0±4.6b	558.0±17.4a	111.3±5.5b	595.3±18.9a	5.0±0.4b
	12%	60.0±2.0c	538.0±33.2a	80.7±2.1c	546.3±30.1a	6.7±0.6a
60 minutes	0%	128.7±6.5b	596.3±2. 9c	130.7±6.5a	768.0±9.8c	4.6±0.2c
	4%	135.3±18.2a	704.0±58.9b	122.3±4.6a	865.3±89.8b	5.8±0.4c
	8%	133.3±4.2ab	901.7±25.7a	104.3±4.0b	1021.3±17.6a	8.7±0.5b
	12%	89.7±5.5c	897.3±28.2a	77.0±3.6c	898.3±28.2b	11.6±0.8a
90 minutes	0%	137.3±7.6a	670.3±32.0c	127.0±2.0a	866.7±40.6b	5.3±0.3d
	4%	151.0±9.5a	841.3±44.9b	119.7±4.2a	995.7±40.5b	7.1±0.4c
	8%	140.3±14.6a	1045.7±42.5a	100.3±10.5b	1174.3±8.5a	10.5±1.5b
	12%	94.7±11.9b	1034.0±71.9a	72.3±4.9c	1043.0±69.1b	14.3±0.5a

Means carrying same letters in columns for particular dough rest time are non- significantly different;
DPP=Date Pits Powder.

Table 2b) - Extensograph data of Hard Red Spring wheat flour blended with different levels of dates pits powder of 80 mesh particle size.

Dough rest Time	DP Level	Energy (cm^2)	Resistance to extension (BU)	Extensibility (mm)	Max (BU)	Ratio No.
30 minutes	0%	104.3±4.9a	456.3±42.5b	136.3±5.0a	570.7±57.6a	3.3±0.45c
	4%	104.3±8.0a	579.0±30.6b	116.7±7.2b	666.3±23.0a	5.0±0.4c
	8%	79.0±5.3b	666.0±36.0a	87.3±3.8c	675.3±4a	7.7±0.5a
	12%	63.0±6.2c	539.7±30.9b	84.7±5.1c	540.7±30.6b	6.4±0.4b
60 minutes	0%	128.7±6.5a	596.3±2.9d	130.7±6.5a	768.0±9.8c	4.6±0.2c
	4%	140.7±21.5a	851.3±21.1c	111.3±11.6b	1002.0±26.8b	7.7±0.7b
	8%	119.3±4.9ab	1089.3±108.1a	85.7±3.8c	1119.3±98.6a	12.8±1.8a
	12%	102.3±5.1b	977.7±29.6b	81.0±2.6c	990.3±35.6b	12.1±0.3a
90 minutes	0%	137.3±7.6a	670.3±32.0c	127.0±2.0a	866.7±40.6b	5.3±0.3d
	4%	146.0±19.1a	986.3±90.5b	103.7±6.7b	1147.0±132.6a	9.5±0.9c
	8%	117.0±19.2a	1273.0±108.9a	75.7±7.0c	1281.7±108.04a	16.9±2.0b
	12%	115.7±16.8a	1163.7±54.1a	79.7±7.2c	1170.7±63.1a	14.7±0.7a

Means carrying same letters in columns for particular dough rest time are non significantly different.

eters help describe gluten strength and dough extensibility characteristics of flour for a wide range of products. Extensograph test can also evaluate the effects of ingredients and fermentation time on dough functionality. The addition of fiber, such as non-starch polysaccharides, was observed to either positively or negatively influence wheat flour dough properties. Although non-starch polysaccharides are less viscous, its higher molecular weight might allow a better interaction with starch–gluten complex thus improve dough properties. Addition of water-insoluble non-starch polysaccharides was shown to decrease dough extensibility (KULP and BECHTEL, 1963). However, addition of water-soluble non-starch polysaccharides increased both resistance to extension and extensibility in a way similar to the effect observed with dough improvers (BLOKSMA, 1971). The resistance to extension (RE) of the control flour has significantly increased ($p \leq 0.05$) at the 4 and 8% DPP levels, where the RE of the 12% DPP dropped relative to 8%, but it was still significantly higher than the control (Table 2a and b). The different behavior of the 12% was also noticed during the discussion of the Farinograph data possibly due to the presence of oil as well as the fiber. The behavior of the 60 and 80 mesh was similar except that the 80 mesh showed higher RE values. RAO *et al.* (2007) reported similar behavior of water soluble non-starch polysaccharides related to RE of dough. Dough extensibility (DE) has significantly and linearly dropped as a function of DPP level (Table 2a and b), where the 60 mesh kept DE at higher levels compared to the 80 mesh.

Table 3 - Rapid visco-analysis data of wheat flour blended with different levels of dates pits powder of 60 or 80 mesh particle size.

	Wheat flour + Date pit powder 60 mesh			
	0%	4%	8%	12%
Peak Viscosity	3457±77.1a	3076±3.5b	2718±14.8c	2473±15.6d
Breakdown	1353±76.4a	1214±120.2ab	1125±26.2b	1038±61.5b
FinalViscosity	3916±24.4a	3500±24.7b	3101±29.7c	2780±15.6d
Set back	1813±129.4a	1639±99.0ab	1508±41.0bc	1345±61.5c
Peak Time	6.2±0.10a	6.2±0.18a	6.1±0.04a	6.03±0.14a
Peak Temp	68.125±0.46a	68.4±1.06a	68.5±0.07a	68.15±0.64a
	Wheat flour + Date pit powder 80 mesh			
Peak Viscosity	3457±77.1a	3040±6.4b	2709±89.8c	2370±103.2d
Breakdown	1353±76.4a	1238±50.2a	1056±57.3b	997±59.4b
FinalViscosity	3916±24.4a	3452±29.7b	3075±73.5c	2666±83.4d
Setback	1813±129.4a	1650±73.5a	1422±41.0b	1293±39.6b
Peak Time	6.2±0.10a	6.165±0.05a	6.24±0.05a	6.07±0.00a
Peak Temp	68.125±0.46a	68.175±0.53a	69.27±0.11a	68.92±0.67a

Means carrying same letters in rows are statistically non significant.

3.5. Rapid Visco-Analyzer (RVA)

The Rapid Visco-Analyzer (RVA) data showed that flour peak viscosity (PV) was significantly dropped ($p \leq 0.05$) as a function of DPP content in the blends. The cause of PV drop could be attributed to the less flour content in the blend, but looking at the extent of drop in PV, it is apparent that some of the drop is due to DPP presence (Table 3). For instance, the addition of 4% DPP should reduce PV by 4% due to less starch in the blend i.e., PV of flour was 3457 Centipoise (cP) should drop to 3318, but it dropped to 3076 indicating that 242 cP (3318-3076) is due to DPP presence. The 3318 cP drop is the theoretical drop of the control by 4% due to the absence of flour and the 3076 cP is the actual RVA reading of the sample. The presence of 8% and 12% exhibited a drop of 462 and 569 cP due to DPP. CHAISAWANG and SUPHANTHARIKA (2006) and GONERA and CORNNILLION (2002) reported a mechanism in regards to PV decrease at higher xanthan gum. This can be accredited to xanthan gum capacity to completely cover starch granules and alleviate granules association and restrict swelling as shown by the confocal microscope and scanning electron microscope. The limited granules swelling resulted in lower peak viscosity. Previous reports showed increase in PV of rice starch blended with okra gum which could possibly be due to adequate granules swelling and okra gum ability to promote granule association which resulted in higher gum concentration located in the continuous phase (ALAMRI *et al.*, 2012). In the presence of DPP, the drop in PV can be interpreted by its ability to prevent starch granules association

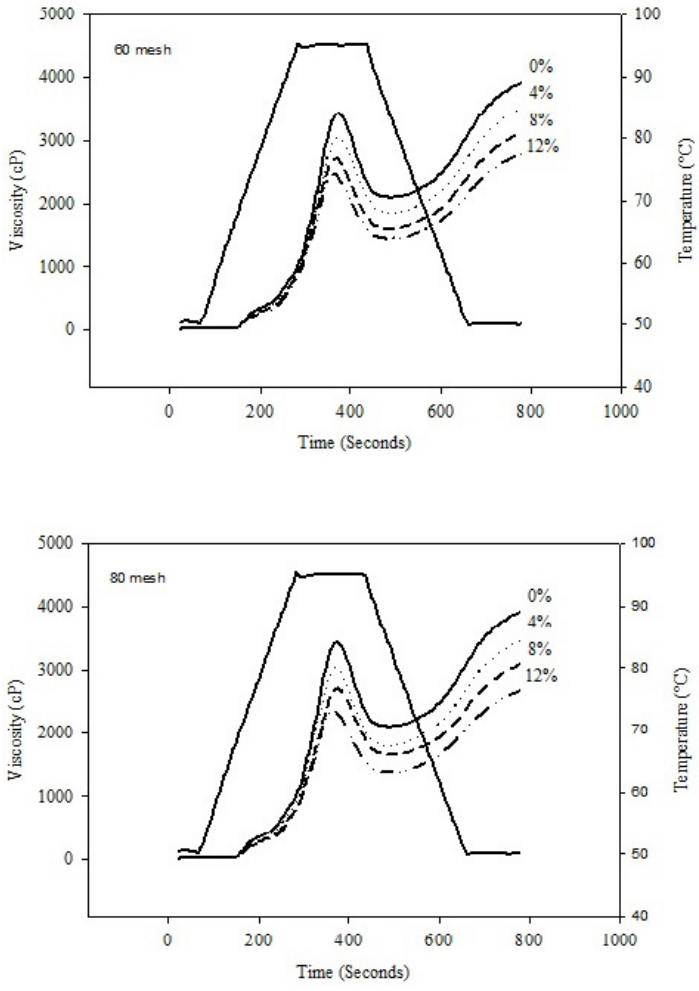


Fig. 2 - Rapid visco analyzer profile of wheat flour blends with different levels of date pits powder of 60 and 80 mesh particle size.

and reduce swelling by covering starch granules (Table 3 and Fig. 2). DPP effect can be attributed to two factors, the presence of soluble fiber that might prevent granules association or the presence of oil that could prevent granules swelling (AZIZI and RAO, 2005), thus reducing the peak viscosity. The effect of DPP on the starch setback (SB) could be discussed in the same manner as PV, where the overall significant drop in SB was shown to be due both, absence of starch and action of DPP (Table 3). The effect on SB due to the 60 mesh DPP was 101.5, 159.9, and 250.4 cP for the 4, 8, and 12% DPP, respectively. The 80 mesh DPP exhibited 90.5, 245.9, and 302.4 cP for the 4, 8, and 12% DPP, respectively. The larger surface area of the 80 mesh DPP appeared to reduced SB more than the 60 mesh (Table 3). The final viscosity showed comparable trends as the PV and SB.

The data in Table 4 illustrate values for hardness of wheat flour gels and blends containing 4, 8, and 12% of the 60 mesh DPP. Hardness is the force required to compress a food between molars and defined as force necessary to achieve given deformation. An interesting observation is the fact that the firmness of the control gels was slightly lowered by the 8 and 12% DPP. Although the RVA peak viscosity, setback, and final viscosity were significantly reduced by DPP, gel hardness appears to be less influenced by DPP presence. This can be attributed to the fact that gel hardness test was done at room temperature and RVA data was collected at temperatures higher than 50°C and under mixing. In essence, changes in starch gelatinization parameters are due to the presence of components other than starch in the control (wheat flour), but DPP components appeared to make up for the lost amylose due to less starch and maintained gel hardness closer to the control. In other words, the replacement of wheat flour with DPP caused reduction of starch in the blend which in turn reduces amylose content in the sample (amylose is main controller of gel hardness). Previous researchers reported the addition of guar gum among other gums to wheat flour and noticed that guar gum maintained gel hardness closer to the control in a replacement study (BRENNAN *et al.*, 2008). The effect of DPP on gel hardness was similar to guar gum, where other gums, such as Arabic gum, caused softer gel. The texture of gels could not be correlated with their final or peak viscosity and appeared to be independent of pasting characteristics observed by the RVA. The 80 mesh DPP showed significant reduction on gel hardness at all three replacements levels (4, 8, and 12%), while all three levels required same deformation force (Table 4). It is apparent that DPP particle size influenced gel characteristics as it did on the final quality of bread as a similar trend reported by MOHAMED *et al.* (2010) regarding the correlation between gel characteristics and final bread quality. Cohesiveness, which is the strength of internal bonds making up the body of the gel, exhibited no significant change in the presence of DPP, whereas springiness, which is ratio of the height when the sample springs back after the compression compared to the maximum deformation, showed no significant change compared to the control. Adhesiveness is the work necessary to overcome the attractive forces between the surface of the food and the surface of other materials with which the food comes into contact. In the presence of DPP, adhesiveness was significantly higher at 8 and 12% DPP indicating stronger gel body, while gumminess was not significantly changed. The 80 mesh DPP exhib-

Table 4 - Gel texture analysis of wheat flour blended with different levels of dates powder of 60 and 80 mesh particle size.

	Wheat flour + Date pit powder 60 mesh			
	0%	**4%**	**8%**	**12%**
Hardness (g)	110.50±10.61a	110.00±10.61a	102.00±0.00a	97.00±4.24a
Cohesiveness	0.47±0.01a	0.47±0.01a	0.43±0.04a	0.47±0.00a
Springiness(mm)	10.95±3.04a	8.90±3.04a	9.30±1.27a	10.15±0.07a
Adhesiveness (mJ)	0.75±0.07a	0.65±0.07ab	0.80±0.14b	1.25±0.21ba
Gumminess (g)	51.38±4.15a	51.15±4.15a	43.35±3.61a	45.59±1.99a
Chewiness (g)	562.64±110.67a	455.24±110.67a	403.155±88.71a	462.74±23.46a
	Wheat flour + Date pit powder 80 mesh			
Hardness (g)	110.50±10.61a	91.00±1.41b	83.00±1.41b	81.00±1.41b
Cohesiveness	0.47±0.01a	0.47±0.01a	0.48±0.02a	0.49±0.00a
Springiness(mm)	10.95±3.04a	9.25±0.64a	9.80±0.28a	9.90±0.14a
Adhesiveness (mJ)	0.75±0.07a	0.95±0.21a	0.85±0.21a	1.05±0.21a
Gumminess (g)	51.38±4.15a	42.32±1.30b	39.43±2.43ba	39.69±0.69b
Chewiness (g)	562.64±110.67a	391.41±38.97a	386.37±34.99a	392.93±1.25a

Mean carrying same letters in rows are statistically non significant.

Table 5 - Volume and instrumental color of bread prepared from wheat flour blended with different levels of dates pits powder of 60 or 80 mesh particle size.

	Wheat flour + Date pit powder 60 mesh			
	0%	**4%**	**8%**	**12%**
Bread volume (cm3)	663.47±11.58a[1]	509.13±3.93b	496.16±8.56b	370.62±3.14c
L*[2]	91.80±0.03a	78.26±0.02b	71.78±0.01c	66.75±0.01d
a*[3]	-0.44±0.03d	4.70±0.01c	6.29±0.00b	7.53±0.01a
b*[4]	6.41±0.01d	7.53±0.01c	7.60±0.03b	8.40±0.01a
	Wheat flour + Date pit powder 80 mesh			
Bread volume (cm3)	663.47±11.58a	585.54±17.89b	509.32±10.12c	375.39±2.65d
L*	91.80±0.03a	77.31±0.01b	72.11±0.01c	65.75±0.00d
a*	-0.44±0.03d	4.74±0.01c	6.40±0.03b	7.68±0.01a
b*	6.41±0.01d	7.74±0.01c	8.46±0.01b	8.89±0.01a

[1]Predicted mean values followed by the same letter within a row are not statistically significant; significantly different based on overlap of the 95% confidence intervals.
[2]L* = lightness, higher values indicate lighter color.
[3]a* = redness.
[4]b* = yellowness; higher color intensity is indicated by higher values.

ited no significant change on the cohesiveness, springiness, and adhesiveness (Table 4). Gumminess of the 80 mesh containing samples was significantly reduced ($p \leq 0.05$). Overall, softer gel compared to the control is due to the absence of amylose due to less starch in the blends, while more influence of the 60 mesh could be due to the less surface area that may allow more control on water mobility thus gels exhibit harder texture than the 80 mesh.

3.6. Bread quality

One of the good measures of wheat flour quality as well as the effect of added ingredients on the final bread quality characteristics is the bread loaf volume. The presence of DPP (both mesh sizes) significantly reduced bread loaf volume due to gluten dilution by DPP. In addition to other components, DPP powder contained around 52% fiber, 11% oil. Due to their structure, fiber absorbs a large amount of water, therefore interfering with gluten development during dough mixing. Lack of completely developed gluten has a direct effect on dough formation, mixing time, and bread final quality. In general, the greatest drop in loaf volume was at highest DPP content (Table 5). The presence of high oil and soluble fiber, in the formulation used here, appeared to have effect on the dough mechanical properties which was tested at (30°C) during Farinograph testing and not during the baking process. This could partially explain the higher dough stability results which did not match with the lower loaf volume for the same dough. Since the baking process is a hydro-thermal process, the behaviour of the dough system with altered formulation must be different at the Farinograph temperature than at the baking temperature. In addition, the critical role of starch on the final loaf volume is well documented, because gelatinized starch forms a gel that maintains the final loaf volume, whereas less starch in the bread formulation could lead to lower loaf volume. One of the ways to deal with the low loaf volume was to add vital gluten so that to counter the negative effect of DPP as reported in previous work (MOHAMED *et al.*, 2010), but that will increase the cost as well as it will darkens the color of the bread because of increase in Millard reaction due to higher protein content. Considering the dark colored DPP in the blends, it was expected that bread color will be darker than the control (lower L* value). Similarly, samples with more DPP powder will be darker. The bread crust data in Table 5 showed just that, where the L* value decreased significantly and in accordance to the DPP powder content, where the 12% blend was the darkest. The crumb showed similar pattern with significant differences between blends. The redness (a*) and yellowness (b*) values of the crumb have significantly increased relative to the DPP content compared to the control and in agreement with DPP level (Table 5). The mesh size of DPP appeared to have less influence on bread color.

Bread staling is one of the important characteristic of stored bread, where bread aroma becomes less desired by consumers. Staling can be quantified by determining bread hardness (texture). In the course of storage, amylose and amylopectin retrogradation are believed to be the main cause of staling. Monoacylglycerols complexation with amylose was found to be effective in reducing staling (WILLHOFT, 1971). So that to ascertain the effect of

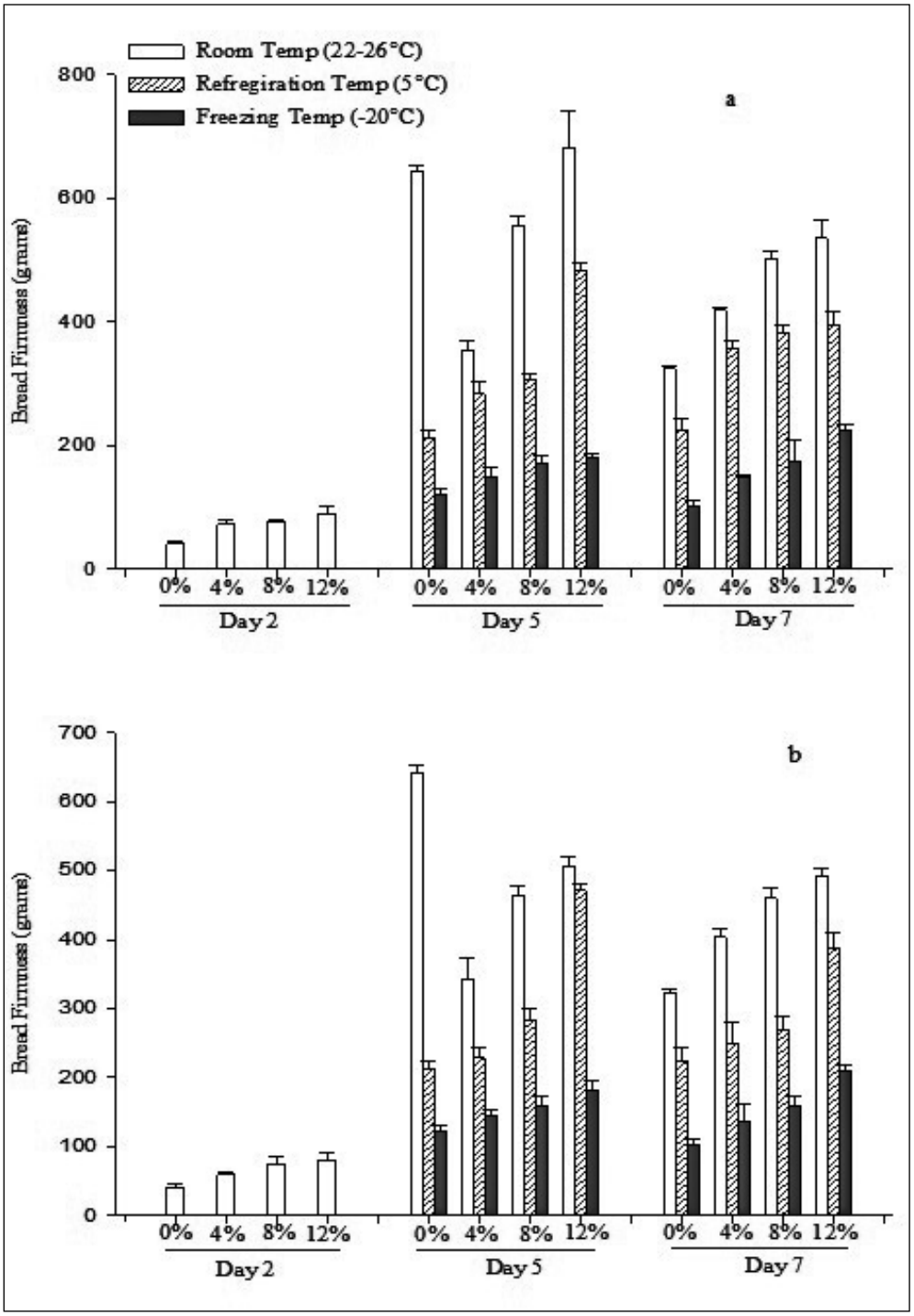


Fig. 3 - Firmness quality of breads made from wheat flour blends with different levels of date pit powder of 60 and 80 mesh particle size after 2, 5 and 7 days of storage at room temperature (25°C), 5° and -20°C (Statistical significance is shown in Table 6).

storage time and temperature on bread firmness, testing was done at 25, 4.0, and -20°C for 2, 5, and 7 days. The time and temperature were chosen to mimic supermarket's storage conditions. On the first 2 days of storage only room temperature was tested for the control and the blends because the 4°and -20°C did not equilibrate at the respective temperature. Significantly at $p \leq 0.05$, the control (0% DPP) exhibited the lowest firmness compared to the blends (Fig. 3 and Table 6). In addition to the increase in firmness of the control with time, the five days indicated uneven firmness increase while the 7 days indicated gradual increase as a function of DPP level. Lower temperature reduced bread firmness at both 5 and 7 days storage time, but higher DPP content lead to higher firmness. The ANOVA results shown in Table 6 also showed that higher DPP content increased bread firmness due to the

Table 6 - Texture analyzer (50 kg cell load) bread firmness data of wheat flour blended with different levels of dates powder of 60 and 80 mesh particle size. This table is the detailed statistical analysis of the data presented in Fig. 3.

	60 mesh									80 mesh								
	2 days[a]			5 days			7days			2 days			5 days			7 days		
% DPP[b]	25[c]	4	-20	25	4	-20	25	4	-20	25	4	-20	25	4	-20	25	4	-20
0	c	-[d]	-	c	c	c	d	c	c	c	-	-	d	c	c	d	b	c
4	b	-	-	b	b	b	c	b	b	b	-	-	c	c	b	c	b	b
8	b	-	-	a	b	a	b	b	b	b	-	-	b	b	b	b	b	b
12	a	-	-	a	a	a	a	a	a	a	-	-	a	a	a	a	a	a

[a]storage days;
[b]% date pits powder;
[c]storage temperature °C;
[d]samples were left to equilibrate and not tested on the first 2 days; p≤0.05.

high fiber content. It is also worth mentioning that lower storage temperatures lead to statistically insignificant difference between samples with different DPP content (Table 6). Elevated amounts of fiber had direct effect on water migration from other bread components, such as protein and gelatinized starch, thus causing bread stiffness. This moisture distribution and trapping by fiber are the cause of higher firmness. Bread enriched with soluble fiber is one of the popular products in the baking industry, where high soluble fiber and protein content were found to reduce bread firmness (MOHAMED *et al.*, 2008). It is apparent from Fig. 3 and Table 6 the greater influence of temperature on bread firmness than storage time. The effect of particle size of DPP showed that bread with 80 mesh exhibited lower firmness compared to 60 mesh, but the overall trend of bread firmness was similar in the case of 60 and 80 mesh. The 0% DPP blend stored at 25°C showed the least firmness, while the greatest firmness recorded was the control stored at room temperature for 5 days. It is noticeable that, blends with 12% DPP exhibited significantly higher firmness within the same storage time.

4. CONCLUSIONS

Even though fiber enriched bread is considered specialty bread, the final bread loaf volume was as good as the control. The presence of DPP increased the fiber content of the 8 and 12% blends to higher than the recommended levels by FDA for health claim. The rheological properties of the blends represented by Farinograph and showed that mechanical properties are a good predictor of the blends baking performance. This bread is a readily obtainable source for the daily recommended soluble fiber intake per bread serving.

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PAPER

THE USE OF A MODIFIED ATMOSPHERE FOR THE STORAGE OF CHESTNUT FRUITS

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ABSTRACT

The commerce of fresh chestnuts deserves special attention due to the postharvest storage processes that are intended to guarantee quality and shelf life maintenance. The aim of this work was to store fresh chestnuts for a maximum period of 90 days at 1 (±1°C) and 90-95% relative humidity (RH) using a modified-atmosphere (MA; 80% CO_2) technology. The hybrid variety Bouche de Betizac (*C.sativa* Mill. x *C.mollissima* Blume) and the European chestnut Garrone Nero (*C.sativa* Mill.) were stored in pallet bags in a CO_2-enriched (80%) atmosphere. To maintain the MA, two different types of materials a traditional polyethylene film (120 μm) and a biodegradable film derived from starch (180 μm) were used.

Each treatment was compared with chestnuts treated with a cold-water bath (sample test) and stored under normal atmosphere (NA) conditions.

Postharvest storage was evaluated based on chestnut respiration, the gas composition (%) of the storage environment, the water content (%), the marketable fruits (%) and the starch content (%).

A high CO_2 level in the storage environment decreased the metabolic activity of the chestnut fruits, which, in general, exhibited a physiology unlike that of dried fruits, both varieties exhibited a low water content (%) under MA storage conditions. The starch content appears to not be influenced by the treatments used in postharvest. Thus, the use of a modified atmosphere is an interesting alternative to a cold-water bath for storing fresh chestnut fruits.

- Keywords: chestnut, storage, modified atmosphere, quality, respiration, starch -

1. INTRODUCTION

After remaining static for some years, the global production of chestnuts has grown since the late 1980s. Currently, the main European producers are Italy, Turkey and Portugal, followed by Greece, France and Spain (FAOSTAT, 2010).

Similar to other major agricultural products, the chestnut market is influenced by the overwhelming importance of the retailers, which affect the organization of the entire supply chain. As a consequence of this modern agrifood practice, new commercial and technological advances are required to meet the present needs of the industry.

Fresh chestnuts have a short shelf life (NAZZARO *et al.*, 2011), and the fruits are highly perishable because of their high carbohydrate (starch and simple sugars) and moisture contents.

Thus, to improve the market, it is essential to offer a product that maintains a high standard of quality after a long period of storage.

The postharvest management of chestnut fruits is closely related to the physiology of the fruits, which is similar to that of a seed (BREISCH,1993). Relative to other nuts, the chestnut embryo is very porous, and its water content tends to decrease quickly during storage. Like all seeds, the chestnut produces little ethylene (<0.01 $\mu L/kg^{-1}h^{-1}$); however, the synthesis of this hormone may increase as a result of stress, such as attacks by pests (insects and/or fungi),which can induce the early germination of the seed (JERMINI *et al.*, 2006).

HARDENBURG *et al.* (1986) have reported respiration rates equal to 2.5-3.5 $mLCO_2\ kg^{-1}h^{-1}$ at 0°C and 7.5 $mLCO_2\ kg^{-1}\ h^{-1}$ for temperatures of +20°C.

Decreases in quality may be caused by moisture loss and the consequent fermentation processes due to continuous gas exchange with the environment via the porosity and high metabolic activity of the fruits in the days immediately following detachment (GIACALONE and BOUNOUS, 1993). Studies on the effect of a cold-water bath treatment on chestnut metabolism have been conducted in an effort to plan the ideal timing and method of application and to define the appropriate technological parameters for fruit storage postharvest (BOTONDI *et al.*, 2009). As with the majority of fruits and vegetables, the maintenance of high quality throughout the postharvest supply chain and a long shelf life for chestnuts depend on the integrated control of several factors, such as the moisture content in the fruit tissues, the storage temperature, the relative humidity (RH) and the presence of oxygen and insect controls (MENCARELLI, 2004).

Published data indicate that the ideal storage conditions for fresh fruits are from −1° to −2°C (ROUVES and PRUNET, 2002; JERMINI *et al.*, 2006) and 90%RH (MENCARELLI, 2004). The management of the environmental gas composition is also reported to be a good complement to a low-temperature postharvest method (KADER, 2001).

Along with traditional chestnut treatments such as hydrotherapy (cold-water baths) and thermo-hydrotherapy (warm-water baths) (JERMINI *et al.*, 2006), techniques for the storage of large quantities of fresh chestnuts have been developed. Controlled-atmosphere (CA) storage maintains the product in CO_2-enriched (10-40%) and O_2-depleted (3-5%) environments for the entire postharvest period (60 days). This treatment slows the metabolic activity of fruits and vegetables and delays sprouting postharvest (MENCARELLI, 2004; WANG *et al.*, 2008). In contrast to CA systems, modified-atmosphere (MA) technologies can be more flexible in extending the benefits of the CO_2 increase for improving the shelf life of a larger number of fresh products during their distribution and storage (LEE *et al.*, 1996).

The present study was conducted to evaluate the quality of a fresh chestnut storage method for Bouche de Betizac and Garrone Nero, two typical chestnut varieties of Cuneo (Italy), during storage in MA at a low temperature for 90 days. This postharvest storage method was evaluated based on the respiration rate of the fruit, the evolution of gases (O_2 and CO_2) inside the storage environment, the water content (%), the fruits' marketability (%) and the starch content (%).

2. MATERIALS AND METHODS

2.1 Plant material

Bouche de Betizac and Garrone Nero chestnuts were obtained from the Bassi Vivai commercial orchard (Piedmont- Italy). These varieties are representative of the Cuneo area. The fruits were picked by hand during the end of September at the ripe stage of maturity and immediately transferred to the laboratory under cold conditions. Damaged and mouldy fruits were eliminated (by hand) prior to the analysis.

The chemical characteristics of the fresh fruits for both varieties are shown in Table 1.

2.2 Storage methods

The experimental storage units consisted of a pallet bag (thermically sealed at the base) of two different types of film, polyethylene (120 μm) and a biodegradable film (180 μm). According to ASTM F 2622-08 and ASTM F 2476-05, the oxygen (O_2) and the carbon dioxide (CO_2) transmission rates, which were measured at 38°C and at 90% RH, were, 2,642 [$cm^3/(m^2*24$ h)] and 10,654 [$cm^3/(m^2*24$ h)], respectively,

Table1 - Chemical characteristics of the fresh Bouche de Betizac and Garrone Nero chestnuts at harvest.

Content (unit of measure)	Bouche de Betizac	GarroneNero
Energetic value (kcal/100 g)	299	310
Energetic value (kJ/100 g)	1.258	1.307
Protein (g/100 g)	5.18	4.87
Carbohydrate (g/100 g)	56.59	57.46
Sugars (g/100 g)	13.06	13.95
Glucose (g/100 g)	0.59	0.84
Fructose (g/100 g)	0.67	0.73
Sucrose (g/100 g)	10.53	11.39
Lactose (g/100 g)	N.R.< 0.10	N.R.< 0.10
Maltose (g/100 g)	1.27	0.99
Starch (g/100 g)	43.53	43.51
Fat (g/100 g)	2.17	3.59
Fiber (g/100 g)	15.98	14.33

for the polyethylene film and 645 and 5,984 [$cm^3/(m^2*24$ h)], respectively, for the biodegradable film. All pallet bags were injected with 80% CO_2, 15% N_2 and 5% O_2. The gas composition of the MA was chosen based on a previous report (ANELLI and MENCARELLI, 1988), with some modifications. The system used to modify the atmosphere was a flow-through type that operates with high and low pressure sides using carbon dioxide and oxygen gas. The air present in the envelope was partially removed and substituted with the desired gas mixture. A manual timer regulated both the time of evacuation and filling with the gas. There was one pallet bag (with a volume of 480,000 cm^3) for each treatment. The fruits were stored for a maximum period of 90 days in a cold room 1 (±1°C) and at 85-90%RH. Each treatment was compared with a control stored under a normal atmosphere (NA) and treated with a cold-water bath (JERMINI *et al.*, 2006) (approx.+15°C), maintaining a chestnut/water ratio of 1:3. For the trials in MA, the fruits were placed in a plastic box (5 kg per box) and wrapped with different pallet bags according to the scheme in Table 2.

2.3 Methods of analysis

2.3.1 Respiration rate

Before the storage period, the respiration rates (mg kg^{-1} h^{-1}) were determined for both sample varieties that were (treatment Bw and Gw) and were not (treatment B and G) subjected to the cold-water bath. Chestnuts (100 g with three replicates) of both varieties were placed in airtight glass jars (1,000 mL) with a lid containing a rubber septum in the middle for withdrawing gas. Monitoring was performed at 1 (±1°C).

The rates of oxygen consumption (RO_2) and carbon dioxide production (RCO_2) for the fruits were measured using a gas chromatograph (GC Varian 450, Italy) equipped with a capillary column (Molsieve 5A PLOT 30 μm× 30 m × 0.53 mm) and the closed-system method (FONSECA *et al.*, 2002). This method presents some limitations (FONSECA *et al.*, 2002) but is a simple and rapid method to initially screen for varieties of interest.

After 90 days of storage, 100 g of chestnuts (three replicates) of both varieties and both treatments were placed in airtight glass jars (1,000 mL), with a lid containing a rubber septum in the middle to withdraw gas, to evaluate the residual metabolic activities of the fruits (using the same method described above). The respiration rates were not determined immediately; however, the fruits were maintained for 24 h under the NA at 20°±1°C.

2.3.2 Headspace composition

The carbon dioxide and oxygen concentrations inside the pallet bag were measured using a portable CO_2 and O_2 gas analyzer (PBI Dansensor, Italy). The composition values were measured 24 hours after the constitution of the pallet bags to verify the tightness of the bags and every 15 days over the entire period of the trial. The same air volume was maintained in the pallet across the trial period because the analyzer introduced the same quantity of air that it

Table 2 - Treatments for chestnuts stored at 1 (±1°C).

Treatment	Variety	Storage unit
Bw	Bouche de Betizac	Water bath (sample test)
2B	Bouche de Betizac	Modified-atmosphere (MA) pallet wrapped with polyethylene (PE) film at 80% CO_2
3B	Bouche de Betizac	Modified-atmosphere (MA) pallet wrapped with biodegradable film at 80% CO_2
Gw	Garrone Nero	Water bath (sample test)
2G	Garrone Nero	Modified-atmosphere (MA) pallet wrapped with polyethylene (PE) film at 80% CO_2
3G	Garrone Nero	Modified-atmosphere (MA) pallet wrapped with biodegradable film at 80% CO_2

removed for the analysis. Each value is the average of three measurements and is expressed as a percentage.

2.3.3 Determination of water content

At 0, 30, 60 and 90 days, ten chestnut fruits (three replicates for each treatment) were cut, and the peel was removed. Each sample was weighed, placed in an oven at 60°C for 72 hours and then weighed again. The difference in weight corresponded to the water content and is expressed as a proportion of the fresh weight (%).

2.3.4 Determination of marketable fruits

The chestnuts (100 fruits per replicate (three replicates) for each treatment) were peeled, and the healthy nuts were selected for visual analysis (to detect mould species). Marketable fruit was calculated as the percentage of healthy fruits.

2.3.5 Determination of starch

The control used for the starch evolution for these two varieties is preliminary. For this reason, only the cold-water bath treated chestnuts (Bw and Gw) and fruit stored under MA in the polyethylene bag (treatments 2B and 2G) were compared.

Soluble sugars were extracted from each sample after grinding the peeled chestnut fruits lacking the episperm and incubated at 60°C for 12 h.

Samples (2 g) of flour were diluted in 50 mL HCl to 1.128%. After stirring at 100°C for 15 minutes, 10 mL of the internal standards Carrez 1 (CH_3COO_2Zn and CH_3COOH) and Carrez 2($K_4[Fe(CN)_6]$), which were diluted in 250 mL of distilled water, were added to a final volume of 100 mL. The optical rotation of the resulting solution was measured using an optical polarimeter (digital model POLAX Atago 2-LSAVATEC- Italy), and the starch content (g/100 g) was calculated using the following equation (EC, 2009):

$$\text{Starch content (g/100 g)} = [2{,}000 \times (2.5 \times P')/P] - [(2{,}000 \times 0.0010)/(\alpha)D20°C],$$

Where:

P = the sample weight
P'= the rotatory power of the solution
$(\alpha)D20°C$ = the specific rotatory power of the pure starch matrix analyzed.

2.4 Data analysis

All statistical analyses were performed using SPSS for Windows (Version 17.0). The obtained data were treated using a two-way analysis of variance (ANOVA), and the means were separated using Tukey's test ($P \leq 0.05$). It was possible to perform parametric tests for the percentages because the sample sizes were identical.

3. RESULTS

The conditions under which this monitoring was performed had the aim of simulating the supply chain of the product after storage. In fact, before being included in the circuit for commercial distribution, the fruits undergo a process of stasis at stores to be processed and packaged for the consumer unit.

3.1 Respiration rate

In accordance with BOTONDI *et al.* (2009), Table 3 indicates that the respiration rate was lower for those fruits treated with the cold-water bath (Bw and Gw) than the untreated fruits (B and G).

After 90 days of storage, the measured respiration rate (Table 4) indicated a low residual metabolic activity for the Bouche de Betizac and Garrone Nero for all the considered treatments. In particular, for the Bouche de Betizac, the lower value was for the Bw treatment (0.49 RCO_2 – 0.53 RO_2), whereas there were no differences between the pallet bags (2B and 3B). For the Garrone Nero, the cold-water bath (Gw) and storage in the polyethylene bag (2G) showed similar values.

Table 3 - Respiration rates of fresh chestnuts before storage in the modified atmosphere (MA).

Treatment	24 h at 1 (±1°C)	
	RCO_2 (mg kg^{-1} h^{-1})	RO_2 (mg kg^{-1} h^{-1})
B	11.92	15.12
Bw	28.82	29.54
G	22.42	22.89
Gw	27.86	28.27

Table 4 - Respiration rates after 90 days of storage in the modified atmosphere (MA).

Treatment	90 days of storage at 1 (±1°C) + 24 h in NA at room temperature	
	RCO_2 (mg kg^{-1}h^{-1})	RO_2 (mg kg^{-1} h^{-1})
Bw	0.49	0.53
2B	0.81	0.81
3B	0.79	0.81
Gw	0.18	0.24
2G	0.18	0.22
3G	0.67	0.73

Table 5 - Pallet bag headspace composition.

	O_2% Days of storage																					
Treatment	0		1		15		29		30		31		45		59		60		61		75		90	
2B	5.0	±0.1	8.2	±0.4	9.1	±0.3	6.3	±0.4	5.0	±0.1	3.6	±0.3	10.1	±0.4	9.3	±0.5	5.0	±0.1	4.4	±0.5	13.4	±0.5	12.8	±0.4
3B	5.0	±0.1	17.5	±0.5	14.4	±0.5	16.3	±0.3	5.0	±0.1	2.2	±0.5	14.4	±0.3	14.3	±0.5	5.0	±0.1	5.5	±0.4	16.3	±0.4	17.7	±0.8
2G	5.0	±0.1	2.2	±0.3	12.6	±0.4	9.2	±0.5	5.0	±0.1	2.1	±0.3	2.2	±0.4	16.3	±0.6	5.0	±0.1	4.0	±0.5	14.1	±0.5	16.8	±0.3
3G	5.0	±0.1	18.1	±0.5	12.0	±0.5	17.1	±0.4	5.0	±0.1	2.5	±0.5	18.9	±0.4	19.0	±0.4	5.0	±0.1	5.5	±0.4	14.5	±0.5	6.9	±0.5
	CO_2% Days of storage																					
Treatment	0		1		15		29		30		31		45		59		60		61		75		90	
2B	80.0	±1.2	52.0	±0.5	12.2	±0.5	12.3	±0.5	80.0	±1.2	72.0	±0.6	11.3	±0.5	11.0	±0.5	80.0	±1.2	69.0	±0.8	12.0	±0.4	9.5	±0.3
3B	80.0	±1.2	11.0	±0.7	5.7	±0.3	3.4	±0.4	80.0	±1.2	80.0	±0.7	5.7	±0.3	5.5	±0.5	80.0	±1.2	66.0	±0.7	4.2	±0.4	2.4	±0.5
2G	80.0	±1.2	79.0	±0.7	21.7	±0.4	10.4	±0.5	80.0	±1.2	78.0	±0.5	78.0	±0.4	12.0	±0.4	80.0	±1.2	72.0	±0.8	25.0	±0.5	17.0	±0.5
3G	80.0	±1.2	15.0	±0.6	7.9	±0.3	3.5	±0.3	80.0	±1.2	76.0	±0.5	2.3	±0.4	1.4	±0.4	80.0	±1.2	65.0	±0.7	5.5	±0.5	3.4	±0.4

3.2 Headspace composition

To maintain a high level of CO_2 inside of the pallet bag, the pallets were injected every 30 days with the initial gas mixture (80% CO_2:15% N_2:5% O_2). Table 5 shows the trend of the gas in the headspace of the bag. In the case of biodegradable film, after the first 24h, there was a decrease in the CO_2 values to the 10-15% range for both varieties, and the samples placed in polyethylene had the highest CO_2 values after 29 days.

3.3 Water content

At harvest, the fruits treated with the cold-water bath had the highest value (Table 6), likely owing to water absorption during the treatments, as previously demonstrated by BREISCH (1993). The treatment and duration of storage had a significant effect on the water content of both varieties of fruit.

After 90 days of storage, the Bouche de Betizac, particularly those fruits stored under MA in the polyethylene bag, had the highest water content values (2B),whereas for the Garrone Nero, all treatments under MA had the highest water content values (2G and 3G), with these values likely influenced by the high RH (95%) inside of the pallet bag.

3.4 Marketable fruits

The quality of the chestnuts is also related to defects and disease, and good postharvest management is important to reduce the incidence of mould contamination and growth. *Penicillium* spp. and *Mucor* spp. were the most common moulds (JERMINI *et al.* 2006) found on the chestnuts of both cultivars in this study, whereas infections of living larvae were not found. The proportion of mouldy chestnuts among the diseased fruits was generally high. Treatment and duration of storage (Table 7) had significant effects on the development of the moulds. For both chestnut varieties, the percentage of healthy fruits decreased as the storage time increased.

Table 6 - Bouche de Betizac and Garrone Nero water contents (%).

			Days of storage		
Chestnuts variety	Treatment	Harvest	30	60	90
Bouche de Betizac	Bw	53 a[a]αβ[b]	50 bβ	58 n.s.α	54 bαβ
	2B	48 bβ	54 aαβ	57 n.s.α	60 aα
	3B	48 bγ	50 bγ	53 n.s.αβ	55 bα
Garrone Nero	Gw	50 aα	46 aαβ	48 n.s.αβ	45 bβ
	2G	40 bγ	39 bγ	49 n.s.β	59 aα
	3G	40 bγ	40 bγ	48 n.s.β	57 aα

[a]For each variety, the means in a column followed by different letters are significantly different at $p \leq 0.05$ according to Tukey's test;
[b]For each treatment, the means in a row followed by different letters are significantly different at $p \leq 0.05$ according to Tukey's test.

Table 7 - Bouche de Betizac and Garrone Nero marketable fruit (%).

			Days of storage		
Days of storage	**Treatment**	**Harvest**	**30**	**60**	**90**
Bouche de Betizac	Bw	62 n.s.[a]α[b]	41 aβ	18 aγ	13 aγ
	2B	60 n.s.α	25 bβ	19 aβγ	16 aγ
	3B	60 n.s.α	22 bβ	10 bγ	9 bγ
Garrone Nero	Gw	67 bα	34 bβ	26 bβ	25 bβ
	2G	74 aα	56 aβ	55 aβ	46 aβ
	3G	74 aα	42 bβ	47 aβ	24 bγ

[a]For each variety, the means in a column followed by different letters are significantly different at $p \leq 0.05$ according to Tukey's test;
[b]For each treatment, the means in arrow followed by different letters are significantly different at $p \leq 0.05$ according to Tukey's test.ù

Table 8 - Bouche de Betizac and Garrone Nero starch (%).

			Days of storage		
Days of storage	**Treatment**	**Harvest**	**30**	**60**	**90**
Bouche de Betizac	Bw	56 n.s.[a]α[b]	53 n.s. β	44 bγ	52 b β
	2B	55 n.s. n.s	56 n.s. n.s	53 a n.s.	54 a n.s.
Garrone Nero	Gw	53 n.s.α	49 n.s. α	44 b β	43 n.s. β
	2G	50 n.s.n.s.	47 n.s. n.s.	50 a n.s.	45 n.s. n.s

[a]For each variety, the means in a column followed by different letters are significantly different at $p \leq 0.05$ according to Tukey's test;
[b]For each treatment, the means in a row followed by different letters are significantly different at $p \leq 0.05$ according to Tukey's test.

In general, the Bouche de Betizac was more sensitive to damage than the Garrone Nero. In fact, a reduced quantity of marketable fruits was observed starting at 60 days. However, under the 3G treatment, low-quality fruits were only observed for the Garrone Nero at 90 days.

3.5 Starch

Among the treatments, differences for both varieties were detected during storage (Table 8). For the Bouche de Betizac, the treatments significantly affected the starch value at the end of the storage treatment. During the MA treatment, no differences could be detected during storage. For the Garrone Nero, the duration of storage, but not the treatment, had a significant overall effect on the starch content. Starch was metabolized faster in the cold-water bath (Gw), but the reduction in the starch content was of the same magnitude for all the treatments.

4. CONCLUSIONS

This study demonstrates the validity of MA treatments, which have the dual effect of maintaining fruit quality and protecting fruits from spoilage due to moulds. A low temperature 1 (±1°C) and high CO_2 level (80%) regimen was particularly efficient for the storage of the Bouche de Betizac and Garrone Nero fruits compared to the traditional cold-water bath treatment. Even if the cold-water bath (water absorption and anaerobic conditions) reduces the respiration rate of the fruits immediately after harvesting, the MA, with high levels of CO_2, reduces the metabolic activity of the fruits during the entire storage period. For the management of pallets in a modified atmosphere, the polyethylene bag demonstrated the best features. The biodegradable film used here displays a low barrier to humidity and gas (DONHOWE and FENNEMA, 1994; FORSSELL *et al.*, 2002).

It is important to emphasize that the 2011 (in Piedmont, Italy), the chestnut growing season was characterized by high rates of fungal diseases, particularly in early varieties at the time of picking, and that such infections certainly negatively affected the storage potential of these fruits.

For this reason, the current amount of marketable fruits at the time of picking is not more than 70%. In particular, the MA treatments maintained significant quantities of marketable Garrone Nero.

The total starch content, which was determined using an optical polarimeter, is in agreement with values reported in the literature (JERMINI *et al.*, 2006; PEREIRA-LORENZO *et al.*, 2006; DE VASCONCELOS *et al.*, 2009; BOTTA *et al.*, 2008; CHENLO *et al.*, 2009). This method represents an objective, simple, rapid, accurate and economical alternative for measuring the starch content of fruits. As shown by NOMURA *et al.* (1995), starch degradation is influenced by the

varietal component, and, in general, the starch content of the Garrone Nero fruit is always lower than that of the Bouche de Betizac fruit during all periods of storage.

The choice between postharvest treatments depends on various factors, including the business structure and the requirements of the market. Therefore, the proposed modified atmosphere storage conditions for pallets of fruits area promising strategy for satisfying the demand for chestnuts, particularly during the offseason, and for diversifying their consumption.

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PAPER

NEOPHOBIA IN FOOD CONSUMPTION: AN EMPIRICAL APPLICATION OF THE FTNS SCALE IN SOUTHERN ITALY

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ABSTRACT

Through the Food Technology Neophobia Scale (FTNS) proposed by Cox and Evans (2008) this work investigates the role of consumer attitudes to food technology in determining the probability of purchasing innovative products by using a set of commonly purchased food.

Six food categories with different processing levels were analyzed. Consumer choices were empirically modeled through a simultaneous system of three equations. The results confirm the strength of the FTNS scale, reflecting the dichotomy between neophobia and neophilia attitudes even in southern Italy, characterized by a very different socio-cultural context from that analyzed by Cox and Evans. The model highlights the role of both consumer attitudes to technology and socio-demographic characteristics in determining the probability of buying innovative products, providing some initial elements to define ideal customer profiles for the products considered.

- Keywords: food technology, consumer attitude, neophobia, psychometric scale -

INTRODUCTION

Diet is the most intimate form of consumption: according to anthropologists, eating means incorporating, taking food inside oneself, within the confines of one's own body (FISCHLER, 1990). For this reason, diet and food are also characterized by a simultaneous connotation of demand for novelty (neophilia) and by a great caution, at times aversion, concerning the new, the unknown (neophobia). Roughly speaking, it could be said that food choice is a seemingly simple, but in fact very complicated, behaviour, influenced by many interacting factors (KOSTER, 2009).

Consistent with this approach, modern food consumption seems to follow two different and contrasting patterns: while there is a growing demand for modernity (functional foods, convenience foods, healthy foods, such as low calories and low-sodium foods), on the other hand consumers are increasingly demanding for naturalness (organic foods, natural foods, local products and typical products) (GOLDSMITH and HOFACKER, 1991; ROZIN *et al.*, 2004; ROZIN, 2005; ROLLIN *et al.*, 2011; O'BRIEN, 2012).

In recent years, consumers' fears of modern food technologies have been well documented and several studies have analyzed consumer acceptance of new technologies, pointing out that consumers show high levels of concern toward novel food (TOURILA *et al.*, 2001; MATIN *et al.*, 2012; BONANNOXS, 2012; BARRENA and SÁNCHEZ, 2013), genetic modification (HU *et al.*, 2004, 2006; LARUE *et al.*, 2004) and more generally toward foods that have undergone a considerable degree of industrial processing (ROZIN *et al.*, 2004; ROZIN, 2005; ROZIN, 2006). Also factors that can influence consumer acceptance have been analyzed, focusing on the risk-benefit perception, socio-demographic and economic factors, the role and source of information and finally public trust (MACFIE and MEISELMAN, 1996; SIEGRIST, 2000; SIEGRIST *et al.*, 2005; VISSCHERS *et al.*, 2007; COSTA-FONT *et al.*, 2008; ROLLIN *et al.*, 2011; FREWER *et al.*, 2011; CHOE and CHO, 2011; COX *et al.*, 2012; UELAND *et al.*, 2012). At the same time, different psychometric scales were set and tested to analyze consumer attitudes towards modern and new technology (GOLDSMITH and HOFACKER, 1991; STEENKAMP and BAUMGARTNER, 1995; SPARKS and SHEPHERD, 1994; EISER *et al.*, 2002; KIRK *et al.*, 2002; COX *et al.*, 2007; COX and EVANS, 2008). These scales can be particularly useful in identifying population segments that have greater or lesser neophobia-neophilia attitudes, thus enabling identification of early adopters of innovative products (COX and EVANS, 2008; EVANS *et al.*, 2010b).

Psychometric techniques involve several sets of statements, usually formulated through the use of qualitative methods, such as focus groups and in-depth interviews. The statements are submitted to the sample of respondents called to express their degree of agreement-disagreement using Likert scales. This process generates a large amount of information and can be synthesized in correlation and covariance matrices. Consumers' preferences, analyzed by means of factor analysis, multidimensional scaling, cluster analysis, path analysis and structural equations, provide the main outcome of the psychometric analysis (WEBER *et al.*, 2002; SIEGRITS *et al.*, 2008).Thus the adoption of psychometric scales may provide accurate analysis of the consumer preferences structure (BEKER, 1976; LUSK and BRIGGEMAN, 2009).

At least two psychometric applications concerning consumer food preferences are of great relevance today: first the perception of risk and how it affects the markets in the event of a hazard; secondly, the acceptance of novel food technologies.

This paper, following the second line of research, investigates the relationship between the acceptance of modern technology and consumption choices.

The latest proposed scale for the assessment of the neophobia-neophilia dichotomy is the Food Technology Neophobia Scale (COX and EVANS, 2008; EVANS *et al.*, 2010b). The FTNS is presumed to be a better instrument for predicting consumers' willingness to try novel food technologies than an earlier food neophobia scale (FNS) (PLINER and HOBDEN, 1992) because of its specific focus on technology rather than food (MATIN *et al.*, 2012).

It has been mainly applied on specific food technologies or on food specifically related to new and sophisticated technological processes (COX and EVANS, 2008; EVANS *et al.*, 2010a). However it seems worth adopting the Cox scale using, first of all, a set of food products instead of food technologies and, secondly, taking into account a set of commonly used products that can be easily ranked by consumers on higher/lower perceived technology content.

This paper wants to contribute to the debate using a psychometric scale, in particular the Food Technology Neophobia Scale (FTNS), to analyze the relationship between attitude toward food technologies, intention and behavior. According to well-known psychological theories (e.g. theory of reasoned action, planned behavior theory), the most important antecedent of people's behaviour is the intention to act in a certain way, which, in turn, is predicted by their attitude towards the target of that behavior (FISHBEIN and AJZEN, 1975; AJZEN, 1991). Attitudes are defined as people's cognitive and affective orientations towards relevant social targets and can be measured directly (explicitly) or indirectly (implicitly).

The paper explores the neophobia-neophilia dichotomy by carrying out an empirical analy-

sis on a sample of 355 people interviewed shortly after their shopping trip to super- and hypermarkets in Campania, a region in southern Italy. A questionnaire was used to obtain responses to the FTNS statements and to collect information about the perception of six food categories with different processing degrees, the perception of naturalness and its role in determining consumer preferences for each food category.

To assess how neophobia-neophilia attitudes affect purchasing intentions, a Principal Component Analysis (PCA) was carried out on the FTNS statements followed by an econometric model. The econometric model was focused on three food categories, i.e. those with the highest degree of food processing, namely functional foods, low-calorie foods, and convenience (ready-to-eat) foods. The willingness to purchase them was related to factors synthesizing consumers' attitudes toward modern technologies (EISER *et al.*, 2002; BÄCKSTRÖM *et al.*, 2004; BRUNEL and PICHON, 2004; HWANG *et al.*, 2005; CRANFIELD *et al.*, 2012).

The article is organized as follows. After a brief review of psychometric scales (section 2), data analysis and the main research findings of the research are presented in section 3. Finally, in section 4 some conclusions are drawn.

2. MATERIAL AND METHOD

2.1 The survey

A field survey using a three-part questionnaire was carried out during summer 2010 on a sample of 355 people interviewed after shopping at super- and hyper-markets in the Italian region of Campania. The survey in Campania region represents a first attempt to validate the FTNS scale in Italy. The 13 items of Cox and Evans scale were, then, translated to Italian.

The sample is a representative distribution of the regional population in territorial and socio-economic terms (Table 1).

The first section of the questionnaire aimed to define the way consumer perceives six food categories with different degrees of processing that were briefly defined prior to the interview: functional, low-fat, frozen and ready-to-eat products, all involving more processing, on the one hand, and products with a perceived lower level of technology content, such as organic, typical and short-chain products, on the other. These last ones were considered in order to better test whether the consumers clearly distinguish between the two food categories as the technological content is concerned. For each type of food, four main items of information were sought:

– whether consumers trusted it;

– a characteristic that would best represent it among a set relating to safety, environmental impact, naturalness and taste attributes;

– the intensity level of the previous attributes, on a scale from 1 to 7 (from minimum to maximum);

– buying frequencies and the willingness to purchase it.

The second section of the questionnaire aimed to assess consumer attitudes toward technology using the 13 statements taken from the work of COX and EVANS (2008), referring to:

the perception of the consumer about technology, its uses and benefits;

the way consumers feel in new situations and behave when facing unknown circumstances;

food habits and the propensity to taste new products.

While COX and EVANS apply the Food Technology Neophobia Scale (FTNS) to a set of different technologies, in our work the same scale is applied to food categories. This is because:

Since consumers are more knowledgeable about products than technologies, they can express their opinions more fully;

Asking a question on technology is more likely to affect the response negatively because of the negative relation between knowledge and risk perception (FIFE-SCHAN and ROWE, 1996);

Finally, the third section collects socio-eco-

Table 1 - Distribution of main socio-economic indicators: comparison of survey sample and the Campania region data.

	Campania region	Sample
Province		
Avellino	8%	10%
Benevento	5%	7%
Caserta	15%	11%
Napoli	54%	54%
Salerno	18%	18%
Gender		
Male	49%	48%
Female	51%	52%
Education		
Primary school	22%	21%
Middle school	29%	27%
High school	22%	28%
Graduation	15%	24%
Legal status		
Married	38%	37%
Separated	2%	2%
Cohabitant	10%	12%
Single	50%	49%
Professional status		
Self employed	5%	9%
Manager	1%	2%
Employee	20%	20%
Student	18%	15%
Housewife	26%	24%
Retired	12%	9%
Unemployed	11%	9%
Precarious/part time	7%	12%

nomic, psychographic and demographic information on the interviewees and their families, and tries to assess their general approach toward the environment, naturalness and traditions.

2.2 Exploratory analysis and Principal Component Analysis

An exploratory analysis and t-tests were carried out on collected data in order to:

Evaluate how the consumers associate each food category to the attributes relating to safety, environmental impact, naturalness and taste;

Investigate general attitudes toward technology and the perception of its benefits and risks analyzing answers to the Cox & Evans psychometric statements.

Moreover, in order to synthesize consumer attitudes toward technologies and more processed products, Principal Component Analysis (PCA) was performed on the scores to the FTNS questions. In fact, PCA is a statistical technique whose basic assumption is that underlying dimensions, or factors, can be used to explain more complex phenomena. Then, the relationship among FTNS statements could give information on some not-directly-observable factors, relating to attitude toward technology that could lead consumers' behaviour. To test whether the observed variables (the FTNS statements in our case) share common factors the Kaiser-Meyer-Olkin measure of sampling adequacy and the Bartlett's Test of Sphericity can be computed. In our case KMO was 0.746 and Bartlett's Test was 853 (df = 70; $p < 0.0001$), indicating that the dataset was factorable.

The analysis was carried out using SPSS (version 18.0). A varimax rotation was used that allows extraction of components that are highly correlated with a low number of variables and hence results in more interpretable factors.

2.3 The econometric model

To formally assess to what extent the perception of risk affects the consumer's decision to buy food products obtained with modern technologies an analytical approach was developed. The work is based on the hypothesis that the intention of purchasing food products with different technological contents mainly depends on the attitude of consumers toward food technologies measured using the FTNS scale.

This hypothesis is developed by means of a three-equation multivariate Probit model where consumer choice to take part in three markets, namely those with the highest degree of food processing (functional food, light products and ready-to-eat products), is related to the neophobia-neophilia attitudes of consumers (CRANFIELD *et al.*, 2012).

The dependent or response variable is represented by the event that the consumer is interested in the purchase and consumption of a set of three processed food products that are based on the use of modern technology, namely functional foods (y_1), low-calorie foods (y_2) and ready-to-eat food (y_3). In empirical terms, the three-equation multivariate Probit model supposes that:

$$\begin{aligned} y_1^* &= (X\beta_1 + Z\gamma_1 + e_1), \\ y_2^* &= (X\beta_2 + Z\gamma_2 + e_2), \\ y_3^* &= (X\beta_3 + Z\gamma_3 + e_3) \end{aligned} \quad (1)$$

with

$$y_1 = \begin{cases} 1 & if \quad y_1^* > 0 \\ 0 & if \quad y_1^* \le 0 \end{cases}$$

$$y_2 = \begin{cases} 1 & if \quad y_2^* > 0 \\ 0 & if \quad y_2^* \le 0 \end{cases} \quad (2)$$

$$y_3 = \begin{cases} 1 & if \quad y_3^* > 0 \\ 0 & if \quad y_3^* \le 0 \end{cases}$$

X is the matrix of exogenous variables relative to the respondent's socio-economic and demographic characteristics, such as education level, income, age, gender, residency and so forth;

Z is a matrix of exogenous variables that include the consumer's attitudes to technology in relation to food (FTNS scale). These last variables are represented by factors extracted from Principal Component Analysis;

β and γ are parameter vectors.

The error terms are assumed to be independently and identically distributed as trivariate normal:

$$\begin{pmatrix} e_1 \\ e_2 \\ e_3 \end{pmatrix} \sim N(0, \Sigma) \quad \Sigma \equiv \begin{bmatrix} 1 & \rho_{12} & \rho_{13} \\ \rho_{12} & 1 & \rho_{23} \\ \rho_{13} & \rho_{23} & 1 \end{bmatrix}$$

Each equation estimates the probability of the consumer purchasing one of the three proposed product categories that are based on the use of modern technology; in this context the $Pr[y_1 = 0, y_2 = 0, y_3 = 0]$ is equal to:

$$\int_{-\infty}^{-x_1\beta_1} \int_{-\infty}^{-x_2\beta_2} \int_{-\infty}^{-x_3\beta_3} \phi_3(e_1, e_2, e_3, \rho_{12}\rho_{13}\rho_{23}) de_3 de_2 de_1 \quad (3)$$

where $\varphi_3(\cdot)$ is the trivariate standard normal distribution and ρ_{ij} is the correlation coefficient between the error terms of the equation, i and j. In the estimation, if the null hypothesis H_0: $\rho_{ij} = 0$ cannot be rejected, this implies that the equations need not be estimated as a system and can be estimated separately[1].

[1] The condition $\rho_{ij} = 0$ can be tested using a LR test. In order to approximate the trivariate normal CDF, we use the GHK (Geweke-Hajivassiliou-Keane) smooth recursive simulator (MARIANO *et al.*, 2000).

The proposed empirical model jointly assesses three hypotheses:

H_1) Consumption intention (y_1; y_2; y_3) might be linked to the consumer's attitude to technology in relation to the food (FTNS factors). This hypothesis can be tested through the estimation of parameters γ, underlying a causal relation of Z on y_1 and y_2 and y_3.

H_2) Consumption intention (y_1; y_2; y_3) might depend on the complex of socio-economic and demographic characteristics that define the profile of the consumers surveyed. This hypothesis can be tested through the estimation of parameters β.

H_3) Consumption intention (y_1; y_2; y_3) might be intercorrelated by the effect of unobserved characteristics or compounding variables. This event can be taken into account and controlled through the estimation of the coefficients ρ (ρ_{12}, ρ_{13}, ρ_{23}).

3. RESULTS

From preliminary analysis of the data a clear dichotomy emerges when confidence in different food types is investigated (Table 2). Over half the interviewees have no confidence in what are perceived as more processed products (functional, low-fat, and frozen and ready-to-eat food). This percentage drops to 10-17% when organic, typical and short-chain products are concerned. The dichotomy is even more evident when scores for different product characteristics are considered. According to the interviewees, organic, typical and short-chain products are all well defined by naturalness and safety attributes, while very few individuals believe that functional, low-fat and ready-to-eat food can be defined as natural.

By means of t-tests (WELCH, 1947) on the differences of scores given to each attribute and for each pair of food products, more information on the way consumers perceive the investigated products can be obtained. First, functional, low-fat and ready-to-eat products are perceived as very different in respect to organic, typical and short-chain categories for each of the attributes considered. Secondly, the consumer is able to distinguish within the category of more processed products, each of which has its own characteristics. For example, functional food is considered more nutrient, rewarding and tasty than low-fat products. Moreover, while organic and typical products differ in terms of taste, safety and environmental impact, a more clear-cut distinction exists between these two categories, on the one hand, and short-chain products, on the other.

In Table 3, the scores represent the mean level of agreement stated on a scale from 1 to 7. In the sample as a whole, a widespread sense

Table 2 - Consumer product confidence and product characteristics.

Food category	Confidence (% of the interviewees)		Product characteristics (mean score)					
	Yes	No	Natural	Safe	Nutrient	Rewarding	Tasty	Environmental friendly
Functional food	56	44	3.3	3.9	4.4	4.5	4.1	3.3
Light products	51	49	3.3	3.7	3.7	3.6	3.8	3.2
Frozen and ready to eat products	46	54	3.3	3.7	3.7	4.3	4.4	3.4
Organic products	79	19	5.8	5.8	5.9	5.8	5.7	5.9
Typical products	88	12	5.9	5.6	5.7	5.9	5.9	5.5
Short chain products	87	13	5.6	5.2	5.2	5.5	5.4	5.5

Table 3 - Level of agreement with the Cox psychometric questions: mean scores on a 1 to 7 scale (1= strongly disagree; 7= strongly agree).

Question Score	
It can be risky to switch to new food technologies too quickly	4.63
New food technologies may have long-term negative environmental effects	4.75
Society should not depend heavily on technologies to solve its food problems	4.71
There are plenty of tasty foods around, so we do not need to use new food technologies to produce more	4.62
New foods are not healthier than traditional foods	4.72
The benefits of new food technologies are often grossly overstated	4.95
New food technologies are something I am uncertain about	4.89
New food technologies decrease the natural quality of food	4.71
There is no sense trying out high-tech food products because the ones I eat are already good enough	4.89
New food technologies give people more control over their food choices	4.15
New products using new food technologies can help people have a balanced diet	4.25
New food technologies are unlikely to have long-term negative health effects	3.69
The media usually provide a balanced, unbiased view of new food technologies	3.00

Table 4 - Factor loading matrix.

	Components			
	1	2	3	4
It can be risky to switch to new food technologies too quickly	0.777	0.122	0.003	-0.141
New food technologies may have long-term negative environmental effects	0.707	-0.045	-0.144	0.026
Society should not depend heavily on technologies to solve its food problems	0.669	0.145	0.123	-0.027
There are plenty of tasty foods around, so we do not need to use new food technologies to produce more	0.479	0.353	-0.341	0.114
New foods are not healthier than traditional foods	-0.136	0.736	0.049	-0.107
The benefits of new food technologies are often grossly overstated	0.141	0.673	-0.120	-0.042
New food technologies are something I am uncertain about	0.447	0.563	0.039	0.124
New food technologies decrease the natural quality of food	0.439	0.514	-0.258	-0.003
There is no sense trying out high-tech food products because the ones I eat are already good enough	0.315	0.510	-0.067	0.395
New food technologies give people more control over their food choices	0.026	-0.091	0.792	0.080
New products using new food technologies can help people have a balanced diet	-0.054	-0.152	0.689	0.250
New food technologies are unlikely to have long-term negative health effects	-0.071	0.193	0.621	-0.432
The media usually provide a balanced, unbiased view of new food technologies	-0.136	0.002	0.167	0.804

of uncertainty on the technology effects emerges, together with the belief that its benefits are overstated. Moreover, the feeling seems to prevail that innovation in the food sector is somewhat futile since traditional products are good enough and healthier.

The PCA carried out on the FTNS statements resulted in a four-factor solution that explains 56% of the whole variance and identifies four thematic areas, quite similar to those already pointed out by COX and EVANS (2008).

In Table 4, the factor loading matrix is presented, which is the basis to interpret the meaning of each component.

The first component explains 25.7% of the total variance and allows identification of the connection between perceived risk and technologies (perceived risk): as the first component increases, shifting from negative to positive values, consumers increasingly perceive technology as a hazardous factor. The component can then be read as a scale of risk. The perception of technology as risky is further characterized by the uncertainty that is associated to food technologies, a statement that is more strongly correlated with component 2.

The meaning of the first component is also linked to the belief that society should not depend heavily on technologies to solve its food problems. Even this statement could in some way be related to risk perception: food security is a strategic matter for society and should not depend upon technologies, which are seen as fragile and unreliable. The statement that "There are plenty of tasty foods around, so we do not need to use new food technologies to produce more" defines an adverse attitude towards technology, adding an ideological connotation to this first component, even if it shows a more transversal correlation with three of the four components.

The second factor extracted (12.2% of explained variance) summarizes the opinion of respondents concerning the usefulness of adopting new technologies and, more generally captures the level of uncertainty (Uselessness of Technology component). When positive, the second component identifies consumers who do not recognize any real benefit stemming from the introduction of new technologies in food sector. This approach is reinforced by the perception of uncertainty ("New food technologies are something I am uncertain about") (HANSEN *et al.*, 2003; FREWER and SALTER, 2003). On the contrary, negative values of the component occur when consumers believe that new technologies can produce more benefit in terms of healthy nutrition, taste and food quality. The third component (9.5% of the total variance) is positively correlated with the perception of benefits of new food technologies in terms of control over food choices; capacity to have a balanced diet and health effects (Benefits and health effects).

Aspects referring to the role of media in conveying information on food technology are shown by the fourth component (Trust in media role). Positive values of the fourth component (8.5% of the total variance) identify those consumers who think that the media usually provide balanced, unbiased information. This kind of trust is particularly important as new food technologies are thought to have long-term negative health effects (negative correlation of the related statement with the fourth component).

The t-test (WELCH, 1947) on the means of the extracted factors scores across groups of consumers according to their socio-economic characteristics provides an initial characterization of consumers' attitudes. Scores of all the components are significantly different according to gender: females have a higher perception of the risk and uncertainty associated with new technologies, and perceive the futility of new products more greatly, in terms of taste and quality. However, at the same time, females believe to a greater extent than males that new food technologies can contribute to diet control and health. Having children is associated with

Table 5 - Descriptive statistics of the variables used in the model – (n = 355).

Variable	Variable description	Mean	Std. Dev
Gender	Assumes the value 1 for male interviewees and 2 for females	1.51	0.50
Education	Educational level of the interviewees	2.21	0.82
Children	Takes the value 1 when there are children under 12 years, 0 otherwise.	0.28	0.45
Income	Categorical variable (1-4) assuming the value 1 for low consumer income and 4 for high consumer income	2.55	0.90
Urban	Identifies the respondents living in urban areas. It takes the value 1 if respondents live in the metropolitan area of Naples, 0 otherwise.	0.53	0.50
Farm holidays	Takes the value 1 when the consumer likes to go on farm holidays, 0 otherwise.	0.56	0.51
Traveling	Takes the value 1 when the consumer likes travelling outside the EU, 0 otherwise.	0.35	0.66
Family size	Takes the value 1 when there are more than 3 family members, 0 otherwise	0.45	0.50
Risk	Summarizes the level of perceived risk associated with the use of technology in the food industry	0	1
Uselessness of Technology	Summarizes the assessment of respondents about the usefulness of adopting technologies and, more generally, captures the level of uncertainty	0	1
Benefits and health effects	Summarizes perception of benefits of modern food technologies in terms of control over food choices; capacity to have a balanced diet and health effects	0	1
Trust in media role	Summarizes the consumer perception that the media usually provide balanced and unbiased information	0	1

Table 6 - Regression results from the multivariate probit.

Equation	Functional food		Light Product		Frozen and ready to eat	
	Coefficients	t	Coefficients	t	Coefficients	t
(Constant)	-2.014***	-4.9	-5.913***	-6.8	-0.195	-0.4
Gender	0.503***	2.95	1.501***	4.79	0.217	0.88
Education	0.009	0.09	-0.812***	-3.4	-0.308***	-2.6
Children	-0.217	-1.1	0.193	0.45	-1.198***	-3.1
Income	0.089	0.97	0.727***	3.41	-0.243	-1.6
Urban	0.871***	5.26	-0.277	-0.7	-0.671***	-2.8
Farm holidays	-0.453***	-2.8	0.169	0.53	-0.643***	-3
Travelling	0.223**	2.07	-0.246	-0.6	0.138	1.29
Family size	-0.18	-1	0.315	0.81	0.541**	2.33
Risk	-0.384***	-4.7	-0.841***	-3.9	-0.113	-1.2
Uselessness of technology	-0.132	-1.6	-0.568***	-2.9	-0.148*	-1.7
Benefits and health effects	-0.006	-0.1	-0.049	-0.3	0.071	0.62
Trust in media role	0.031	0.37	0.191	0.82	0.116	1.08

*significant at the 10% level; **significant at the 5% level; ***significant at the 1% level.

a higher level of risk perception and trust in the role of media (BARRENA and SÁNCHEZ, 2010). Living in an urban area increases the level of risk perception and the level of belief in the benefits and health effects of new technologies, while it is negatively related to trust in the media.

Table 5 reports definitions and the main statistics of the variables in the econometric model. The system estimation results are listed in Tables 6 and 7. Overall, the model shows a good explanatory capacity. Indeed, prediction indexes, which measures the relation between y values correctly determined and total y values observed, are 77.2, 98.3 and 93.5, respectively for system equations 1, 2 and 3.

According to the results, hypothesis H_1 cannot be rejected. The probability of entering the market of proposed food categories depends on consumer attitudes to technology. Nevertheless, the adopted model shows a wide range of results

Table 7 - Model Diagnostics.

Observation	355
LogLikelihood	-243.2
$\rho_{(1/2)}$	-0.409***
$\rho_{(1/3)}$	-0.487**
$\rho_{(2/3)}$	-0.138
Wald $\chi 2$ (d.f)	252.9(36)
Prob>$\chi 2$	0.000
L.R $\chi 2$ (d.f)	11.7(3)
Prob>$\chi 2$	0.008

Table 8 - Average marginal effects of observed consumer attitudes toward technology on consumption probability (%).

	Functional food	Light Product	Frozen and ready to eat
Risk	-9.8	-2.5	-1.1
Uselessness of technology	-3.4	-1.7	-1.5
Benefits and health effects	-0.2	-0.1	0.7
Trust in media role	0.8	0.6	1.2

in terms of the relationship between consumption propensity and consumer attitude.

Specifically, our estimate provides evidence for the existence of a direct relationship between the perception of risk and consumer participation in the market of "Functional" and "Light" products. Moreover, respondents showing greater skepticism toward food technologies and their usefulness are associated with a low probability of buying "Light" and "Frozen or ready to eat" products. By contrast, the third and fourth components were not found to be statistically significant and they do not seem to affect consumer intention. However, the latter two components account for the least explanatory power in terms of the proportion of total variance.

Therefore, the propensity to purchase more processed food products is mostly affected by the perception of risk and by the judgment about the usefulness of technologies. When perceived risk and the feeling of uselessness of technology are high, consumers tend not to participate in the market. The above result is confirmed by the computed marginal effects as reported in Table 8, demonstrating and quantifying the role of the factors for each selected food category.

Among the three products considered, the consumption of functional food seems to be more influenced by the consumer's attitude to technology, presenting a higher marginal impact.

The model results highlight the role of the socio-demographic characteristics of the respondents in determining the probability to purchase innovative products, validating the second hypothesis H_2. The value of the coefficients can provide initial elements to depict the ideal customer profile for the various goods considered. With respect to functional foods, we can assume that a typical consumer is female; she lives in a city, and has a passion for travelling. The typical consumer of light food is also female; she has a high income and her level of education is below average. The consumer of frozen and ready to eat foods has a large family and both the income and the level of education are below average.

The particular behaviour of women, however, is not in contradiction with what we described in the previous section: from cross-comparisons with the presence of children, women seem to perceive more risk, but at the same time, in the absence of children they are more likely to consume functional foods and light products.

The proposed model also confirms the H_3 hypothesis. The correlation coefficients between the error terms among the equations ($\rho_{1/2}$ and $\rho_{1/3}$) are significantly different from zero. Compounding factors influencing consumption of functional food are negatively correlated with the compounding factors influencing consumption both of light products and frozen and ready-to-eat food. This outcome confirms the empirical efficacy in modelling consumer choices simultaneously; it improves the estimation efficiency, including in the analysis the impact of valuable non-observable information.

Since its development the FTNS scale was tested as predictor of the willingness to try foods produced by novel technologies. COX and EVANS (2008) provide information on the FTNS predictive validity by means of Pearson correlation with the willing to try foods produced using novel technologies. Other studies focused on different food technologies (MATIN *et al.*, 2012; EVANS *et al.*, 2010a; CHEN *et al.* 2013). In all these studies the predictor was the total score of the FTNS, while in the current application the predictors were the 4 components extracted from the factor analysis. In this way, the multidimensional nature of the FTNS, is taken into account and it has been possible to analyze the different roles played by the four components in addressing the purchase behaviour toward each specific food category.

5. CONCLUSION

The present study adopted a psychometric scale proposed by COX and EVANS (FTNS) to relate the consumption of specific food categories with the attitude of consumers towards technology.

Our results confirmed the strength of the FTNS scale. First of all, the survey confirms that the psychometric scale used by COX and EVANS can reflect the dichotomy between neophobia and neophilia attitudes. PCA analysis extracted 4 factors: the first two factors (risk and uselessness of technology) are strictly linked to the more or less innovative behaviour of the interviewees.

Consumer choices were empirically modelled through a simultaneous system of three equations. The model highlighted the role of both consumer attitudes to technology and socio-demographic characteristics in determining the probability of purchasing products with a higher degree of processing. It also provided some initial elements to define ideal customer profiles for the products in question. The most important finding concerns the propensity to purchase more processed food products which is mostly affected by the perception of risk and the opinion of the usefulness of technologies.

This evidence may influence future developments of the research in question. The joint use of the COX and EVANS scale and a large set of socio-demographic, behavioural and psychographic data could allow identification of the early adapters segment most willing to join markets for innovative products. This would provide useful insights for marketing strategies, effectively targeting communication techniques and new product launches.

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PAPER

NANOTECHNOLOGY AND ITS APPLICATIONS IN FOOD AND ANIMAL SCIENCE

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ABSTRACT

Nanotechnology has the potential to manipulate matter at the nanometre scale, creating and assembling substances at a molecular level with new and interesting properties. This has offered opportunities for applications in different sectors. Recently, nanotechnology has received particular attention due to its promising applications in animal nutrition, drugs and nutrients delivery, animal reproduction, disease diagnosis and treatment. In the food sector, nanotechnology is applied to the improvement of food packaging, processing, monitoring and the development of food with new functional properties that can respond to the needs of consumers. This review will focus on the advances of nanotechnology in food sector and animal science with particular attention to animal nutrition. The implications for safety and regulation will be also discussed.

- Keywords: nanotechnology, animal science, nanofood, nanoparticles, animal health, safety -

1. INTRODUCTION

1.1 Nanotechnology background and definition

Nanotechnology is a new emerging multidisciplinary field, which is expected to revolutionize both science and society, and provides several long term benefits. In the last years nanoscience was applied to create new tools for molecular biology, new materials in optics, new delivery systems of drugs, new sensors for disease detection. More recently, nanotechnology has received particular attention due to its promising agricultural and food applications. According to the National Science Foundation and National Nanotechnology Initiative (NNI), nanotechnology is the ability to understand, control, and manipulate matter at the level of individual atoms and molecules, as well as at the "supramolecular" level, involving clusters of molecules (in the range of about 0.1 to 100 nm), in order to create materials, devices, and systems with new properties and functions because of their small structure (TANIGUCHI, 1974; DOWLING *et al.*, 2004). These particular features were used from the ancient time. Indeed, nanotechnology have been unknowingly applied from the 4th century BC , when gold nanoparticles (NPs) were used in the manufacture of porcelain in China and Egypt (DANIEL and ASTRUC, 2004) and for medical purposes (such as the treatment of arthritis or the elixir of youth) (READ, 1961). An example of the application of their optical properties was (GONELLA and MAZZOLDI, 2000; PADOVANI *et al.*, 2003) the *Lycurgus* cups, which were made during the Roman empire by using colored glasses containing silver and gold NPs (HARDEN and TOYNBEE, 1959). The cup is characterized by a dichroic effect: the glass is green if the cup reflects light and red if it transmits it. In 1959, the physicist Richard Feynman proposed to study the matter at a molecular scale, stimulating the interest of many people. The famous talk called "there's plenty of room at the bottom" was given by Feynman at the annual meeting of the American Physical Society and is a cornerstone in the short history of the nanotechnologies (FEYNMAN, 1960).

From the beginning of this century, there has been a great increase in scientific publications regarding applications of nanotechnologies (Fig. 1).

The interest in this discipline is due to the unique biological, physical and chemical properties of the Nanomaterials which can be applied to new and useful products (SARGENT and JOHN, 2010). For example, in the second half of the eighties new Nanomaterials such as fullerenes (C60) and carbon nanotubes (CTS) were developed and widely applied. In particular, the interest in CNTs is due to their property to interact with macromolecules and nanostructures of biological interest having similar size such as viruses, nucleotides and DNA, aminoacids, proteins, membranes, including also the water molecules (HAMAD *et al.*, 2002; O'CONNELL *et al.*, 2002; WANG *et al.*, 2002; PAUNESKU *et al.*, 2003; ZHANG *et al.*, 2009). Today nanotechnology has different industrial applications and is more developed in areas such as pharmaceutical sciences, microelectronics, chemistry, material and aerospace sciences (WEISS *et al.*, 2006) (Fig. 2). More recently, nanotechnology has also received considerable attention for its promising agricultural and food applications. In the food sector, nanotechnology has been applied to the improvement of food packaging, processing, monitoring and the development of foods with new functional properties that can respond to the needs

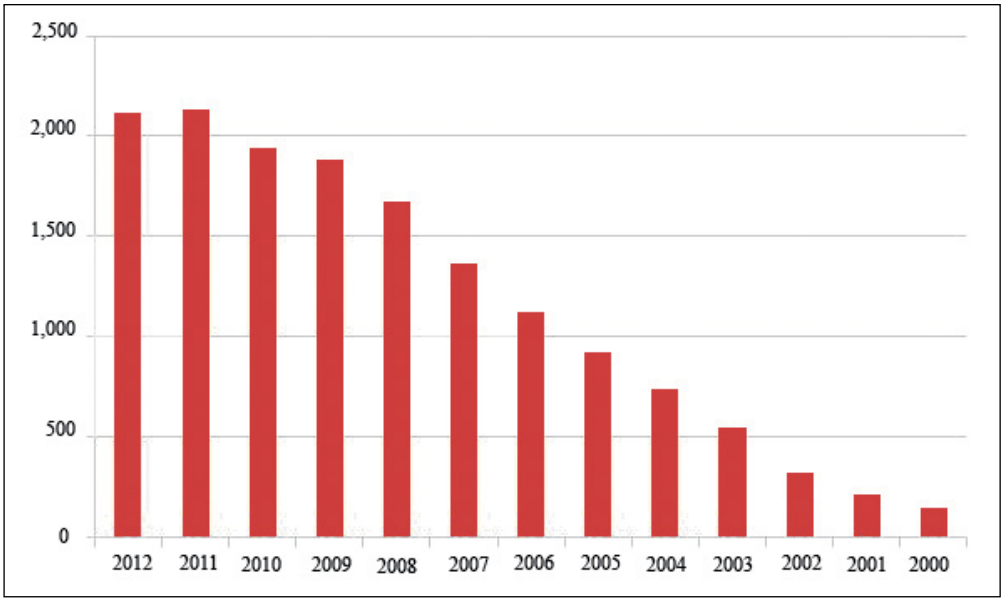


Fig. 1 - Web of Science (search keyword: nanotechnology): Number of publication in the last 10 years.

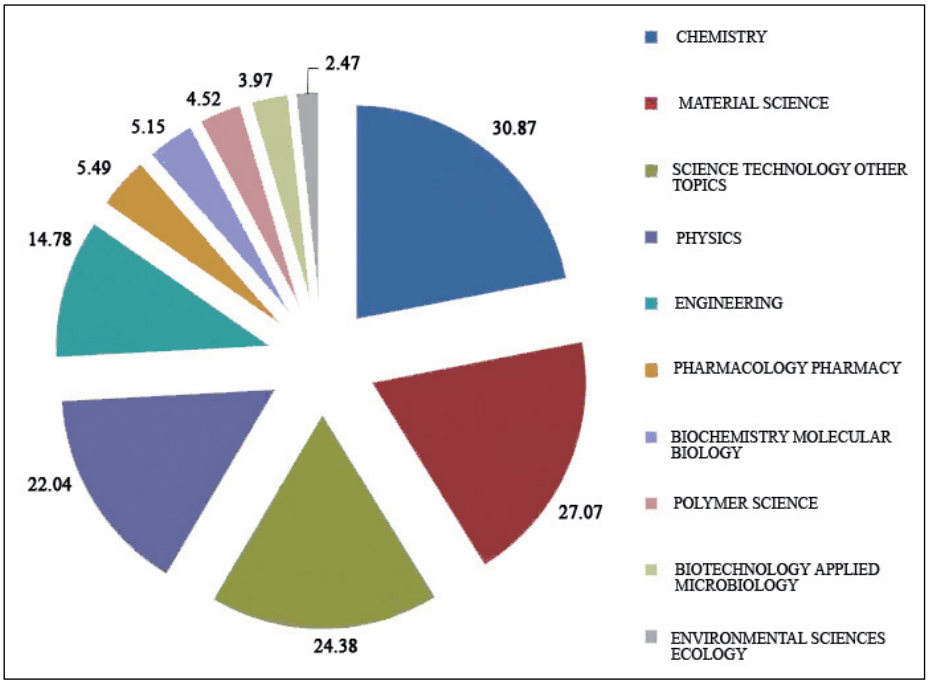


Fig. 2 - Web of Science (search keyword: nanotechnology): % of publication in the last 10 years/research topic

of consumers. The continuous development of this new discipline has led to an increased production and release in the environment of carbon nanotubes (CNTs) and nanoparticles (NPs) by different industries. In this way the population was exposed to high levels of these products that caused severe problems to human health. Therefore, different studies evaluated the toxicity of these compounds at molecular level, although the results were often conflicting (LIU *et al.*, 2012; SHVEDOVA *et al.*, 2012; SAYES *et al.*, 2011). In the next sections, advances of nanotechnology in food sector and animal science will be presented. Their implications for safety and regulations will be also discussed.

2. APPLICATION OF NANOTECHNOLOGY IN ANIMAL HEALTH AND PRODUCTION

For a long period, nanotechnology studies were focused on the medical field and in particular on the evaluation of new drugs *in vivo* (OTILIA *et al.*, 2005). More recently, the research has focused on animals of zootechnical interest. Although there are not many studies on this topic, many advantages can be obtained by applying this technology to animal production (SCOTT, 2005). Several types of nanostructures and NPs have been developed and have revolutionized the approach to animal sciences. Examples of NPs applied to animal sciences are reported in Table 1. In particular, nanotechnologies were applied to the development of novel drug delivery systems and nanosensors for the diagnosis and treatment of diseases. Thus, the effective cure of infections, metabolic diseases, and nutritional disorders was achieved by acting at the nanometer level, Through the control of the matter at the nanometer scale, excellent results in animal health and production were also achieved.

2.1 Administration of medications and vaccines to animals

The health of animals is very important and several efforts have been aimed to afford a high level of protection from pathogens and also avoid infections in humans. Animals are vaccinated by using antigens and adjuvants that stimulate the immune response against a specific virus. However, some adjuvants can produce negative side effects. Nanotechnology represents a promising approach that can allow to reduce such adverse reactions. A new formulation with NPs constituted by calcium phosphate as adjuvant was successfully tested in a murine model for its capacity to induce antiviral immunity (HE *et al.*, 2000). There are also several ongoing studies regarding the encapsulation of vaccines with novel nanocarriers, such as spore based nanovaccines, NPs bound to antigens and synthetic virus-like particles. These formulations allow to transport a drug in the specific site of action with low parental invasion, thus decreasing of the stress of the animal (KALKANIDIS *et al.*, 2006). In this way, the efficacy of the vaccine is increased and the inflammatory response is avoided (SINGH *et al.*, 2007; CAPUTO *et al.*, 2009). At the moment, one of the most important issues of vaccine distribution is the need to maintain the cold chain, which an extension of the good

Table 1 - Overview of NPs and their application in animal science. Revisited by DE JONG and BORM, 2008.

Particle class	Materials	Application	References
Natural materials or derivatives	Chitosan Dextrane Gelatine Alginates Liposomes Starch Vicillin	Drug/Gene delivery	Damascelli *et al.,* 2003 Dyer *et al.,* 2002 Huang *et al.,* 2004 Mura *et al.,* 2011 Murata *et al.,* 1998 Roy *et al.,* 1997
Dendrimers	Branched polymers	Drug delivery	Meredith *et al.,* 2011
Fullerenes	Carbon based carriers	Photodynamics Drug delivery	Yamakoshi *et al.,* 2003
Polymer carriers	Polylactic acid Poly(cyano)acrylates Polyethyleinemine Block copolymers Polycaprolactone	Drug/gene delivery	Du *et al.,*, 1998 Xu *et al.,* 2009
Ferrofluids	SPIONS USPIONS	Imaging (MRI)	Bernardi *et al.,* 2008 Stern *et al.,* 2007
Quantum dots	Cd/Zn-selenides	Imaging *In vitro* diagnostics	Cho *et al.,* 2007 Liu *et al.,* 2011
Various Gold nanoparticles Gold nanorods Gold nanoshells Gold nanocages	Silica-nanoparticles Mixtures of above Solid lipid formulations	Gene delivery	Kawano *et al.,* 2006 Li *et al.,* 2010 Liu *et al.,* 2007 Amarnath *et al.,* 2010 Ankamwar *et al.,* 2005 Salem *et al.,* 2003 Stem *et al.,* 2007

manufacture practice of the pharmaceutical industry (GMP). In order to bypass this requirement, vaccines were formulated in tablets instead of using liquid formulations stored in refrigerator. Moreover, other routes of administration are under evaluation. Nanotechnology represents a promising new approach that allows to improve the vaccine distribution by increasing their shelf-life (PEEK *et al.*, 2008).

2.2 New drug delivery systems

Normally drugs and probiotics are delivered to animals by injection or through the diet. The administration of drugs is realized when the first symptoms appear or in advance, as prophylaxes. Nanodevices can afford a controlled release of drugs , thus avoiding the problem of drug resistance, which arises following the extensive exposure to the drug. Antibiotics administered to animals can reach the consumer. For this reason, there is the need of a technology that could decrease the dose maintaining the same efficacy (HUH and KWON, 2011). In order to achieve this aim, the use of nanoparticles bound to antibiotics was evaluated. According to Huh and Kwon's studies, Ampicilline bound to NPs was as effective as the unbound Ampicilline in reducing the growth of *Salmonella typhimurium* at a 40 times lower concentration of antibiotic. Such a decrease of dose was due to the NPs, which allowed a better and more specific distribution of the drug in the tissue. Controlled delivery systems were also created to deliver and monitor the delivery of vaccines or pharmaceuticals and nutrients in animals (CHEN *et al.*, 2005; MOZAFARI *et al.*, 2006; DE JONG and BORM, 2008). These formulations can be used to deliver drugs in a specific site. Other applications regards the use of NPs for gene therapy (MARSAN *et al.*, 2009; SHARMA *et al.*, 2011), tumor inhibition, as gene delivery vectors or as new engineered nanomaterials for the coating of implants (AJMONE *et al.*, 2007; KATRAGADDA *et al.*, 2010; KUZMA, 2010).

2.3 Nanosensors for diagnosis and treatment of diseases

Nanoparticles can also be used to improve the detection of microbial and chemical contaminants in animals. Examples of this application are the detection of *E. coli* by using a mannose targeting nanobiomolecule that links the pathogen (ZUCCHERI and ASPROULIS, 2012), detec-

tion of *Listeria monocytogenes* in spiked milk (DE LA TORRE *et al.*, 2005; YANG *et al.*, 2007) or Campylobacter (LATOUR *et al.*, 2003; FRANKLIN *et al.*, 2003) frequently found in poultry as contaminant, or polymeric NPs that are able to bind the pathogens in the gut of livestock and then are eliminated in the animal waste through the intestine (KEENER *et al.*, 2006). For the eradication of nematodes such as *Trichinella spiralis*, a frequent contaminant of pork meat, NPs composed of adjuvants, which stimulate the immune response in mice, were used and a decrease of the larvae concentration in the muscle tissue was obtained (DEVILLE *et al.*, 2005). Furthermore, nanosized sensors composed of carbon nanotubes were successfully used to detect toxic compounds and to monitor health and physiological conditions of animals (DRABU *et al.*, 2010). Nanomaterials can be used to create new kits for an early detection of contaminants with high precision. WEI *et al.* (2010) reported a specific application for the rapid detection of melamine in whole milk mediated by unmodified gold nanoparticles.

In biomedical science, one of the current challenges in disease diagnosis is the *in vivo* application of ultrasensitive image analysis of biological targets under noninvasive conditions in order to precisely detect early-stage diseases and monitor the responses to drug therapies. Nanoparticles are now emerging as novel and promising candidates for high-performance molecular imaging agents. Nanostructures, such as shells, quantum dots, NPs, dendrimers and fullerenes, were used to improve potential application in cancer imaging by magnetic resonance imaging (MRI), in order to improve cancer diagnosis and treat target tumors *in vivo* (ZHANG *et al.*, 2008; ROSENBERG *et al.*, 2010).

2.4 Nanotechnology for reproduction

At the moment, there is particular interest in the sustainable production of animals and new solutions are required to obtain an improvement in this field. Nowadays, the use of assisted reproductive technique still presents shortcomings and new technologies, which could improve it, are required (VERMA *et al.*, 2012). In particular, nanotechnologies are used for the monitoring of the fertile period, which is the major problem in farm animals. Nanotubes were applied to the detection of estrus in real time by implanting them under the animal skin. Variations of estradiol levels in the blood of animals were monitored in real time (LIU and WONG, 2007). Nanostructured immunosensors were created in order to detect progesterone in cow milk and evaluate its release during the ovulation period (CARRALERO *et al.*, 2007). The biosensor should allow to detect changes of the physiological level of the progesterone concentration with fertility tests. Silver nanoparticles were evaluated for their antibacterial properties (GARDEA *et al.*, 2003) on chicken embryos. Unfortunately, the treatment was not effective and toxic effects affecting number and size of embryo lymph nodes were found (GRODZIK and SAWOSZ, 2006). Microfluidic devices were developed with the aim of physically mixing sperm and eggs, thus obtaining a cheap, reliable and quick embryos production (STUDNICKA *et al.*, 2009; PATIL *et al.*, 2009). The future plan is the *in vivo* implant of nanocapsules containing the male semen, which should be released in a controlled manner during the ovulation period in order to fertilize an egg.

2.5 Animal nutrition

Emerging evidences indicate that nanotechnology may represent a promising approach to develop new and specific products for animal nutrition. In particular nanotubes linked to nutrients can be administered to animals and released in specific sites, thus allowing the maintenance of high levels for a long time. This approach should avoid the degradation of nutrients and increase their availability (ROSS *et al.*, 2004). Sodium selenite NPs coated with metacrylate copolymers, sensitive to variations of pH, were orally administrated to ruminants and the improvement of selenium absorption was evaluated (ROMEO-PEREZ *et al.*, 2010). Silver NPs and Cu-montmorillonite NPs were used as feed additives to increase the average daily weight gain of pigs (TONG *et al.*, 2007; FONDEVILA *et al.*, 2009). Iron or selenium NPs were successfully tested as feed additives in aquaculture and improvement in animal growth performance was reported (ZHOU *et al.*, 2009). The administration of copper and zinc (HAN and BAKER, 1993; SMITH *et al.*, 1997) as growth promoters in pigs were restricted to the only improvement of animal health status. The use of nanotechnology permits a specific administration of microminerals avoiding their excessive use, which could lead to toxic effects for animals, consumers and environment (FONDEVILA *et al.* 2009 a,b). Minerals, supplements and vitamins are important in animal nutrition; for example iron is essential in animal diet during particular periods of life, such as parasite infestations, gestation and in the early stage of life (ZIMMERMAN, 1980; O' NEILL *et al.*, 2010). However, the administration of ferrous sulfate in food produces a metallic taste due to the increase of rancidity of cereals caused by acceleration of fat oxidation, thus inducing the refuse of food by animals (HURREL, 2002). Ferric phosphate is an alternative to ferrous sulfate but its instability and low availability limit the use as iron source. However, nanoparticles of this compound overcome the stability issue, thus increasing the nutritional value of this source and allowing the administration to animals (FIDLER, 2008; ROHNER *et al.*, 2007). Moreover, nanosized vitamins are used in

poultry and livestock feed. Nanotechnology can also provide applications for medication, nutrients and veterinary care that target specific cases, such as aged animals.

3. APPLICATION OF NANOTECHNOLOGY IN FOOD PRODUCTION

The application of nanotechnology in food systems can lead to several advantages. The food safety has been improved by using sensors for pathogen detection, remote controls of food, smart systems for product tracking, encapsulation of functional food ingredients containing medicines and supplements, which are released in a specific site (WEISS *et al.*, 2006). By mean of nanotechnology, the inclusion of nano supplements or nutrients in food can improve the nutritional value of food or change its taste, color and texture. According to GARBER (2007) and SCRINIS (2007), nanofood can be defined as the manipulation of seeds, feed, pesticides and packaging at the nanometer scale or when nanotechnologies are used during cultivation, production and processing of the food. Due to the possibility to create new useful and interesting products, the food industry has applied this technology to the encapsulation and emulsion of nutrients, probiotics, supplements and to sensor development (FSAI, 2008). In addition to the needs of the industry, the interest of the consumers is to follow the path of the animal and its products throughout all the production and distribution chain giving particular attention to their safety. New compounds for agriculture were also produced by using nanofertilizers composed of hydrogels or zeolites, which improve water catchment in the soil and absorb toxic compounds at the same time (GONZALES-MELENDI *et al.*, 2008). All these possible applications can lead to an improvement of food production linked to a cost effectiveness.

3.1 Application to the food industry

The food industry is the largest manufacturing sector in the world and new technologies are necessary for its innovation. The application of nanotechnology to the food industry involves several benefits, such as products with new properties (less sugars and fats, new textures, colors, nutrients, flavors and tastes), improved food security, processing and traceability, new packaging and products having extended shelf life and improved microbial food safety (AVELLA *et al.*, 2005; CHAUDHRY *et al.*, 2008; GARBER, 2007; RHIM, 2007). It has been estimated that the market of nanotechnology will reach 1 trillion USD by 2015. In particular, packaging is considered the main commercial application of nanotech in food sector. As estimated by Richardson and PIEHOWSKI (2008), nanotechnology will be used in 25% of food packaging within the next decade. Due to the large economic interest, large industries, such as Kraft foods, founded a nanotech consortium (15 University and Research labs) with the aim of obtaining food products that fit the tastes and needs of consumers (SANGUASNSRI and AUGUSTIN, 2006).

3.2 Food safety and decontamination

In term of food safety, one of the main issues is the contamination of cereals with mycotoxins due to its relevance for human/animal health and the economic impact on the global trade of cereal. The FAO has estimated a contamination of 25% of worldwide cereals stockpile by mycotoxins each year with an enormous economic effect (JELINEK *et al.*, 1989; LINDEMANN *et al.*, 1993). Regarding this topic, nanoabsorbents composed of magnesium oxide and embedded by silica nanoparticles has been used as effective adsorbent agents as a way to remove aflatoxins from wheat flour (LUO *et al.*, 2004; MASOERO *et al.*, 2007; MOGHADDAM *et al.*, 2010). SHI *et al.* (2009) reported the use of a modified montmorillonite to decrease the toxicity in feeds of chicks. The development of nanodevices for food safety and preservation and for the detection of biological contaminants of food, such as bacteria, fertilizers or pesticides, is very important (DINGMAN, 2008). Indeed, food pathogens can be detected with nanosensors placed in packaging materials as a sort of electronic noses or tongues, which are able to detect spoilage and contamination of food. These systems allow to detect even just one bacterium located in food in real time with a quick, simple and sensitive procedure (DAS *et al.*, 2009; LILIE and CANTINI, 2011). The advantages of these systems consists in placing thousands of nanostructures in a nanosensor, which is able to detect the presence of any number of pathogens because it can be introduced in tiny places where pathogens hide. Different systems were applied also to drinking water. Recent studies suggest that many of the issues involving water quality could be resolved or greatly improved by using nanoparticles, nanofiltration or other products resulting from the application of nanotechnology. Innovations in the development of novel technologies to desalinate water are among the most exciting and promising targets (SAVAGE and DIALLO, 2005).

3.3 Food processing and monitoring

Food traceability is important in order to follow all steps of the production and distribution of animal products and guarantee their quality. Some procedures are illustrated in SHEFER and SHEFER (2003 a,b) and CHAKRAVARTHI and BALAJI (2010). The application of nanotechnology in

food processing includes the use of nano-ingredients that change texture and taste of food. The dispersibility of additives in food is improved by their encapsulation in nanostructures, such as micelles or liposomes, that can mask the taste of some additives or protect them from degradation (CHAUDHRY and CASTLE, 2011). In this way, the bioavailability of nutrients is increased too, thus reducing the use of additives and preservatives and obtaining a better food storage. Liposomes in particular can be used to encapsulate hydrophobic or hydrophilic ingredients. By adjusting their internal pH, the stability of the substances incorporated is increased. Different materials and shapes can be obtained, such as multiple vesicles with an onion-shaped structure. As illustrated by WEISS (2006), liposomes were successfully used to encapsulate antibacterials, thus obtaining improvement in the inhibition of bacteria growth when compared to the standard methods. This effect may be related to the delivery properties of nano-encapsulated drugs, which are released for a prolonged period of time due to a controlled release (CHEN *et al.*, 2005). Future perspectives of nanotechnology application in food processing are the production of functional food providing health benefit or products tailored to satisfy specific nutritional needs or problems, such as allergies. Recently, omega-3 microcapsules, like many other nano-ingredients, have been added to several food products (MOZAFARI *et al.*, 2006). At the moment, more than 100 nanofoods and others products containing nano-ingredients are sold in the market (Table 2) (FOE, 2008). In these products, nanotechnology has found application in primary production, stock, monitoring and retail level.

3.4 Food packaging

Food packaging represents an important category of nanotechnology application in the food sector. Several nanomaterials have been used in the food packaging for different purposes, such as antibacterial activity (nano-zinc and nano-silver oxide), control of spoilage in orange juice (EMAMIFAR *et al.*, 2011), UV protection (nano-titanium dioxide), gas barrier (plastic polymers with nanoclay), improvement of the mechanical strength (nano-titanium nitride), hydrophobic surface coating (nano-silica), etc. (CUSHEN and CUMMINS, 2012). Thus, the shelf life of the food was increased by improving the mechanical, thermal and gas properties of packaging and using environmental friendly materials (PERCH, 2007; SOZER and KOKINI, 2009). 'Intelligent' food packaging incorporating nanosensors or indicators that monitor the condition of the food have been previously described. These products give an added value to animal products, thus obtaining an overall benefit for the production and distribution chain of food products.

4. RISK ASSESSMENT

Besides the advantages of nanotechnology applications, the possible health hazard linked to increasing exposure to nanotechnology products and the consumption of food produced with this technology must be considered. With the increased production of NPs by many industries, the population may be exposed to high levels of these products, which may have hazardous effects on animals, humans and environment. Nanotechnology may guarantee improvement of hygienic conditions in food processing, good food quality, safety and decrease of foodborne illnesses (CHAUDHRY and CASTLE, 2011 b, BHATTACHARYA *et al.*, 2007). However, the different pathway that nanomaterials can undertake in eukaryotic cells must be evaluated in order to have a better understanding of the correlation between the health hazard of these products and their dimensions (MCKNIGHT *et al.*, 2003). For example, studies reported in the literatures proved that silver NPs, which are commercially used in packaging materials, transfer from the packaging to the food and a certain level of toxicity was demonstrated (ASHARANI *et al.*, 2008; HUANG *et al.*, 2011). The pathway of nanoparticles in the organism is not well known but some studies have demonstrated that NPs can enter in the gastrointestinal tract through trans membrane segments and can cross the placenta (BHATTACHARYA *et al.*, 2011; ELSAESSER and HOWARD, 2012). By interaction with living systems, nanomaterials may be accountable for unexpected toxicity and for this reason their use in food or food packaging may be a reason of health hazard (DAS *et al.* 2009; CHEFTEL, 2011). Other studies (BARCELO and FARRÈ, 2012; IJABADENIYI, 2012) claim that people have been exposed to NPs in the diet for long time and that food ingredients are reduced to the nanometer scale by the digestive processes in order to obtain energy and maintain physiological processes. According to this opinion, the hazard is not due to the dimensions but to the nature of the materials, which can be harmful to human and animals. The toxicity of nanomaterials at molecular level is the topic of more recent studies and the results are often conflicting (LIU *et al.*, 2012; SHVEDOVA *et al.*, 2012; SAYES *et al.*, 2011). At the moment, there is not enough information about the risks for human, animal and environment (DOWLING *et al.*, 2004; WILLIAMS *et al.*, 2005; CASABONA *et al.*, 2010). Several researchers state that regulators do not respond to this issue to safeguard the big companies that are investing on this new technology (BOSSO, 2010). A major concern is related to the consumption of foods containing nano-additives that are indigestible, insoluble and potentially bio-persistent, therefore not beneficial for consumer health. Moreover, the exposure to nanomaterials may constitute a risk for consumer because they may con-

Table 2 - Examples of foods, food packaging and agriculture products that contain nanomaterials (modified by FOE, 2008).

Type of product	Product name manufacturer	Nano content	Purpose
Functional beverage	Oat Chocolate and Oat Vanilla Nutritional Drink Mixes Toddler Health	300 nm particles of iron (SunActive Fe) Solid hydrophobic nanospheres composed of a blend of food-approved hydrophobic materials encapsulated in moisture-sensitive or pH-sensitive bioadhesive microspheres to enable controlled release of active ingredients.	Fortified flavoured waters and milk with vitamin, mineral and other functional ingredients using nanoemulsion technology for incorporation and controlled release of bioactives. Food that can adjust its colour, flavour or nutrient content to accommodate a person's taste or health condition. Nano-sized iron particles have increased reactivity and bioavailability (Fidler *et al*., 2004.)
Food additive	Aquasol preservative; AquaNova	Nanoscale micelle (capsule) of lipophilic or water insoluble Substances	Nano-encapsulation increases absorption of nutritional additives, increases effectiveness of preservatives and food processing aids. Used in wide range of foods and beverages. http://www.aquanova.de
Food additive	Bioral™ Omega-3 nanocochleates; BioDelivery Sciences International	Nano-cochleates as small as 50 nm	Effective means for the addition of highly bioavailable Omega-3 fatty acids to cakes, muffins, pasta, soups, cookies, cereals, chips and confectionery. http://www.nanobiotech.org/
Food additive	Synthetic lycopene; BASF	LycoVit 10% (<200 nm synthetic lycopene)	Bright red colour and potent antioxidant. Sold for use in health supplements, soft drinks, juices, margarine, breakfast cereals, instant soups, salad dressings, yoghurt, crackers etc. http://www.basf-chemtrade.de
Food contact material	Nano silver cutting board; A-Do Global	Nanoparticles of silver	The surfaces of these cutting boards are coated with nano-silver. Pure silver particles only a tiny fraction of a millimeter in size, combat bacteria and kill 99.9% of all germs. This safely protects your sliced food against microbes, and odorous substances won't gain a foothold. http://www.nanotechproject.org
Food contact material	Antibacterial kitchenware; Nano Care Technology/NCT	Nanoparticles of silver	Ladles, egg flips, serving spoons etc have increased antibacterial properties. Nano-coatings are exceedingly water-resistant on outdoor materials; keeping them drier longer. Oil, coffee and other frequent stains belong to the past, if the material is treated with our protective products. http://www.nanocare-ag.com
Food packaging	Food packaging Durethan® KU 2-2601 plastic wrapping; Bayer	Nanoparticles of silica in a polymer-based nanocomposite	Nanoparticles of silica in the plastic prevent the penetration of oxygen and gas of the wrapping, extending the product's shelf life. To wrap meat, cheese, long-life juice etc www.marmarapolimer.com
Food packaging	Imperm (from Nanocor Inc.)	It Is used in multi-layer PET bottles and sheets for food and beverage packaging	Used to minimise the loss of CO_2 from the drink and the ingress of O_2 into the bottle, thus keeping beverages fresher and extending shelf-life. www.nanocor.com
Food packaging	Aegis OXCE (Honeywell)	Polymerized nanocomposite film. The resins are a blend of active and passive nylon using O_2 scavengers and passive nanocomposite clay particles to enhance the barrier properties for retaining CO_2 and keeping O_2 out.	It is an oxygen-scavenging barrier resin formulated for use in co-injection PET bottle applications e.g. beer, fruit juice and soft drinks. http://www51.honeywell.com/sm/aegis/products-n2/aegis-ox.html
Plant growth	PrimoMaxx, Syngenta	100 nm particle size emulsion	Very small particle size means mixes completely with water and does not settle out in a spray tankurf treated results in grass which is healthier, has a more durable blade with a higher tolerance to weather extremes. http://www.syngenta.com
Smart filters	DOW™FILMTEC™ reverse osmosis and nanofiltration elements	The filter consists of a polycarbonate membrane etched with tiny, evenly-sized pores less than 10 nanometers. The thiols spontaneously arrange themselves into a membrane one molecule deep, with all the thiol molecules pointing the same way.	Selective nanofilters that can distinguish molecules based on shape as well as size enabling the removal of toxins or adjustment of flavour. http://www.dowwaterandprocess.com
Water sanitation systems	Nevada-based Altair	It uses 40 nm particles of a lanthanum-based compound which absorbs phosphates from the water and prevents growth of algae	Nanotechnologies makes a water-cleaning product for swimming pools and fishponds called NanoCheck. Besides, nanoscale delivery of weedicides and soil-wetting agents may be very useful for aquatic weed control in large water bodies, and mitigation of stress due to climate change and aquatic pollution. http://www.azonano.com/article.aspx?ArticleID=663
Smart sensors	RFID technology, Sygenta sensors University Innnovation center	Track real time stresses of perishable goods from farm gate to retailer's shelf	Packaging with nanosensors that indicate when a product is compromised and not safe for consumption. http://www.foodproductiondaily.com
Sanitizer Packaging	Nano ZnO Plastic Wrap		Nanoemulsion of vegetable oil, surfactant surrounded by trace amounts of alcohol suspended in water with properties to kill bacteria. Packaging with nanosensors that indicate when a product is compromised and not safe for consumption. Nanoemulsion of vegetable oil, surfactant surrounded by trace amounts of alcohol suspended in water with properties to kill bacteria. http://www.google.com/patents/EP1411913A4?cl=en

tain organic moieties that can carry foreign substances in the blood (MUKUL *et al.*, 2001) and can enter in the gastrointestinal tissue. The exposure to these components of nanomaterials could produce abnormal concentrations of reactive oxygen species and activate an inflammatory response (POWELL *et al.*, 2010). Considering all these risks, an extensive study that correlate the application of nanotechnology in the food packaging with the health hazard must be carried out. At the moment, only few studies have been carried out (DAS *et al.*, 2009), but there is the need to evaluate these nanomaterials by using different assays in order to establish the risk related to their uptake. Furthermore, governments should regulate the commercialization of these products and label them, thus making the consumers aware of the presence of nanofoods in the products (SOZER and KOKINI, 2009). Following these considerations, nanomaterials should be used only after rigorous tests that establish their safety for the environment and the health of humans, fishes and animals (DOWLING, 2004; MEDINA *et al.*, 2007; BLASER *et al.*, 2008; ELSAESSER and HOWARD, 2012).

CONCLUSIONS

Nanotechnology has the potential to revolutionize the field of animal science and food production in terms of diagnostics, discovery of new drugs, drug delivery, vaccine development. The application in food production and packaging can potentially improve the nutritive value, quality and shelf life of several feed and food products with different advantages for the safety of consumers. At the moment, many of these products are either under development or at the experimental stages. At the same time, nanotechnology applications may be accountable for harmful effects for animals, humans and environment. Therefore, one of the main concerns regarding this new technology is the fear of the unknown. For this reason, further investigations are necessary in order to reassure the consumer, fill the knowledge gap about nano-toxicity and, at the same time, improve the risk assessment and evaluate their economic advantages.

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ABBREVIATIONS

Carbon Nanotubes (CNTs), Good Manufacture Practice (GMP), International Standard Organization (ISO), Nanoparticles (NPs), National Nanotechnology Initiative (NNI).

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PAPER

A CHARACTERISTIC OF DIETARY FIBER IN BARLEY AND BUCKWHEAT GROATS AND ITS BILE ACID BINDING CAPACITY

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ABSTRACT

The content of dietary fiber and its fraction in barley (BR) and buckwheat (BW) groats and sorption of selected bile acids were examined. The groats were boiled in water at the water to product ratio of 1.5:1 (v/v). The content of neutral detergent fiber (NDF), cellulose (C), lignin (L) and hemicelluloses (H) was determined according to Van Soest, while the total dietary fiber (TDF), insoluble (IDF) and soluble (SDF) dietary fiber according to ASP *et al.*, (1983, 1996). The sorption of selected bile acids was determined by the colorimetric method. The BR groats were characterized by a significantly higher NDF content in comparison to BW groats, i.e. 6.8 and 5.6%, respectively. The TDF content was higher in the BR groats than in the BW groats, amounting to 13.5 and 8.4%, respectively. Contents of NDF, TDF and fractions of dietary fiber in the groats were lower after boiling. The adsorption of bile acids by plant material depended on the type and concentration of bile acids.

- Keywords: fractions, barley groats, buckwheat groats, dietary fiber, bile acids -

1. INTRODUCTION

Cereals, vegetables, fruits and legume seeds, are a rich food source of dietary fibers that promote various beneficial physiological effects for human health.

Dietary fiber does not constitute a chemically defined compound or a homogenous group of compounds, but it is a complex of heterogeneous substances, such as hemicelluloses, pectins, cellulose and lignin. Moreover, dietary fiber contains also non-structural polysaccharides connected with the endosperm and intercellular space, as well as several other compounds, such as polyfructans, indigestible dextrins, polydextrose, methyl cellulose, hydroxypropyl methyl cellulose and resistant starch (ASP, 1996; DEVRIES, 2001; GUNNES *et al.*, 2012; MEYER, 2004; MILLER JONES, 2000; TOSCH and YADA, 2010).

Dietary fiber and its fraction shows various functions in the human organism and different functional properties, and thus has a different effect in the human intestinal tract (MCCONNELL and EASTWOOD, 1974; PEERAJIT *et al.*, 2012; SILVA *et al.*, 2012; TOSCH and YADA, 2010). Dietary fiber shows different physical and chemical properties like water holding capacity (WHC), water binding capacity (WBC), cation exchange capacity (CEC) absorption of metals, but also affects the rate of passage of chyme through the intestines, lowers blood glucose and cholesterol levels, increases feces mass and binds bile acids (CORNFINE *et al.*, 2010; GÓRECKA *et al.*, 2002; KAHLON and CHOW, 2000).

Depending on the source of its origin, dietary fiber is characterized by varying capacity to bind bile acids and their salts in the intestines, thus contributing to increased excretion of these substances and accelerated cholesterol metabolism. Bile acids may be adsorbed on dietary fiber or dissolved and retained in the water trapped in the fiber (EASTWOOD *et al.*, 1976; TOSCH and YADA, 2010). According to CAPALDI *et al.*, (2009) and DZIEDZIC *et al.*, (2012), pectins and lignin lower blood cholesterol and lipid levels in the higher degree, than cellulose fractions.

Cereals belong to the most concentrated sources of dietary fiber. In cereal products the content of dietary fiber varies considerably and depends on the amounts of individual kernel components they contain. During cereal processing to produce groats the seed coat and the germ are removed, as well as a part of the aleurone layer, depending on the milling grade. Cereal products, including groats due to their contents of biologically active substances, may be a raw material for the production of functional food (BAUBLIS *et al.*, 2000; MEHTA, 2005). Barley and buckwheat groats are widely used in Polish cuisine.

2. MATERIAL AND METHODS

2.1. Material

Grains of barley namely Antek was obtain from Breading Station Danko (Poland) and was used for production of barley groats. Buckwheat grains (*Fagopyrum esculentum* Moench), namely Kora was obtain from Breading Station Palikije (Poland) and was used for production of buckwheat groats.

Groats were boiled in water, at the water to product ratio of 1.5:1 (v/v). The products were lyophilized and next ground in a Cyclotec mill (Foss, Tecator) for 10 seconds. Analyses cholic acid (CA) and deoxycholic acid (DCA) were selected at the concentrations of 1 and 6 mM. The selection of bile acid concentrations (1 and 6 mM) was based on the fact that in the intestines these acids are found at the concentrations from 1 to 12 mM (DZIEDZIC *et al.*, 2012; XIANG *et al.*, 2010).

2.2. Determination of dietary fiber content

2.2.1. Contents of neutral detergent fiber (NDF) and its fraction

Contents of neutral dietary fiber (NDF), acid detergent fiber (ADF), lignin (L) and cellulose (C) were determined by the detergent method according to VAN SOEST (VAN SOEST, 1963; 1967). Starch was digested using thermostable α-amylase (Termamyl 120 L, Novozymes) (MCQUEEN and NICHOLSON, 1979). Contents of hemicelluloses (H) were calculated as the difference between NDF and ADF. The analysis were made using FIBERTEC apparatus produced by Tecator.

2.2.2. Contents of soluble and insoluble fibre

Contents of total dietary fiber (TDF), soluble (SDF) and insoluble (IDF) dietary fiber were determined using the Asp method (ASP *et al.*, 1983).

2.3. Measurement of bile acid sorption

The capacity to bind bile acids (cholic and deoxycholic, Sigma Aldrich, Germany) by groats was determined using the colorimetric method (HUANG and DURAL, 1995). In this method the factor inducing the colour reaction is a furfural solution (Merck, Germany). The principle of the measurement is to determine bile acid concentrations in the supernatant after incubation at the temperature of 37°C and the reaction characteristic for the gastric juice. Analyzed substance (0.5 g) and bile acids (20 mL) dissolved in a phosphate buffer (pH 6.9) were placed in a conical flask. Two samples were prepared: one containing bile acids with no plant material add-

Table 1 - The content of neutral detergent fibre and its fraction composition in groats (g/100 g of product).

Kind of groats	NDF	ADF	H	L	C
Raw BR	6.75±0.09^{c}	2.13±0.20^{b,c}	4.62^{c}	1.43±0.08^{c}	0.70±0.03^{b}
Boiled BR	3.88±0.14^{a}	2.01±0.08^{b}	1.87^{a}	0.47±0.02^{a}	1.54±0.07^{d}
Raw BW	5.63±0.12^{b}	2.21±0.18^{c}	3.42^{b}	1.17±0.05^{b}	1.04±0.05^{c}
Boiled BW	3.75±0.1^{a}	1.82±0.09^{a}	1.93^{a}	1.40±0.03^{c}	0.42±0.01^{a}

$^{a-d}$ Values (means ± SD) within a column with different letters differ significantly at $P < 0.05$ (n = 3).
NDF: Neutral Detergent Fibre: ADF, Acid Detergent Fibre; H: hemicelluloses; L: lignin; C: cellulose; BR: barley groats; BW: buckwheat groats.

ed as a standard sample and the other, containing 0.5 g plant material and 20 mL buffer, as the blank test. Samples were shaken in a water bath at the temperature of 37°C for 2 h and subsequently they were filtered.

A total of 5 mL supernatant were collected for analysis and mixed with 5 mL 70% sulfuric acid. After 2 minutes 1 mL furfural solution (25%) was added (pink colouring appeared after 5 minutes). Absorbance was measured at wave length of 510 nm. Cellulose, binding bile acids to a slight degree, was used for comparison.

2.4. Statistics

All analytical determinations were performed at least in triplicate. Values of different parameters were expressed as the mean ±SD. The results of the study were verified statistically with the Scheffe test at $p < 0.05$ significance level (Statistica 9).

3. RESULTS AND DISCUSSION

3.1. Contents of dietary fiber

The analyzed groats were characterized by differing contents of dietary fiber expressed as NDF (Table 1). Dietary fiber content in raw barley groats was higher in comparison to raw buckwheat groats, amounting to 6.75 and 5.63%, respectively.

The dominant fraction in the analyzed groats was the H fraction, which in BR constituted 4.62%, while in raw BW 3.42%. The analyzed groats contained the smallest amount of the C fraction, whereas BW contained more of this fraction than BR, i.e. 1.04 and 0.70%, respectively. A significant effect was observed of thermal processing (boiling) on dietary fiber content and its fractions. In BR the content of dietary fiber decreased by 42.5%, while in BW by 33.4%, respectively. In boiled BR groats the content of H and L fractions decreased by 59.5 and 67.1%, respectively, while C fraction content increased by 120%. In boiled BW groats the content of H and C fractions decreased by 43.6 and 59.6%, respectively, and the lignin fraction increased by 19.7%.

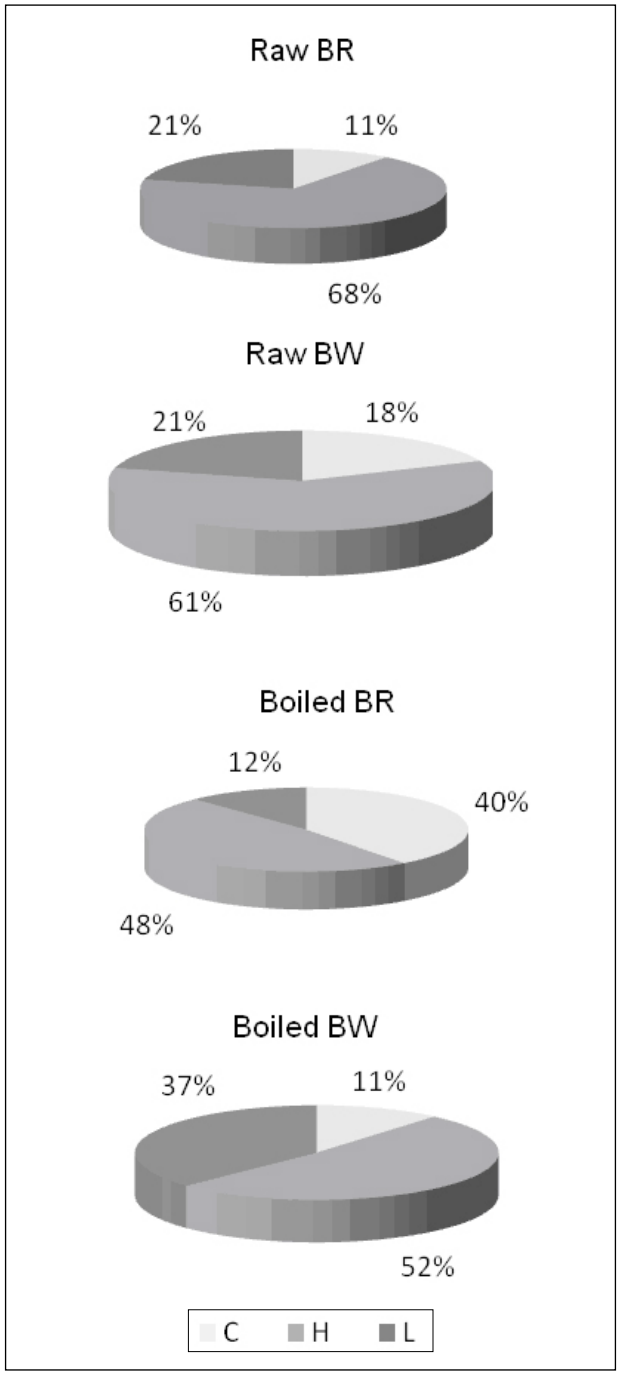


Fig. 1 - Percentage content of fractions dietary fiber of buckwheat and barley groats.
BR: barley groats; BW: buckwheat groats; C: cellulose; H: hemicellulose; L: lignin.

Table 2 - The content of total dietary fibre, soluble and insoluble dietary fibre in groats (g/100 g of product).

Kind of groats	SDF	IDF	TDF
Raw BR	4.74±0.12[d]	8.73±0.20[c]	13.47±0.42[c]
Boiled BR	1.16±0.08[b]	4.61±0.10[a]	5.78±0.13[a]
Raw BW	2.46±0.10[c]	5.94±0.12[b]	8.40±0.20[b]
Boiled BW	0.84±0.05[a]	4.93±0.27[a]	5.77±0.08[a]

[a-d] Values (means ± SD) in a column with different letters are significantly different at *P≤0.05*.
SDF: soluble dietary fibre; IDF: insoluble dietary fibre; TDF: total dietary fibre; BR: barley groats; BW: buckwheat groats.

Table 3 - Proportion between fractions in groats.

Kind of groats	Proportions of fractions			
	H/L	C/L	H/C	SDF/IDF
Raw BR	3.23	0.49	6.6	0.54
Boiled BR	3.97	3.28	1.21	0.25
Raw BW	2.92	0.89	3.29	0.41
Boiled BW	1.37	0.30	4.59	0.17

H: hemicelluloses; C: cellulose; L: lignin; SDF: soluble dietary fibre; IDF: insoluble dietary fibre; BR: barley groats; BW: buckwheat groats.

Percentages of individual fractions in dietary fiber of raw and boiled groats are presented in Fig. 1. Taking into consideration the fractional composition of dietary fiber, the groats demonstrating similar proportions of fractions L and H are raw BR (21 and 68%, respectively) and raw BW groats (21 and 61%, respectively). Boiled groats BR and BW were characterized by similar content of fraction H (48 and 52%, respectively).

In the opinion of many authors the primary components of fiber in cereals are hemicelluloses, constituting 65% of total fiber expressed as NDF, while the percentage of cellulose amounts to less than 25%, lignin content is the smallest in fiber of the analyzed cereals (DZIEDZIC *et al.*, 2012; WESTENBRINK *et al.*, 2013).

Contents of total dietary fiber (TDF), its soluble (SDF) and insoluble (IDF) fractions are presented in Table 2. The analyzed groats showed varying TDF levels, ranging from 5.8 to 13.5% in raw BR. Insoluble dietary fiber constituted the dominant fraction in all groats. In raw barley groats IDF content was 8.7%, while in raw buckwheat it was 5.9%. SDF amounted to 4.7% in raw BR and 2.46% in BW groats. According to DICKIN *et al.* (2011), the content of β-glucans in barley grain varies and ranges from 3 to 7%, although in most cases it is 4-7%. Thermal processing significantly decreased the content of TDF, as well as SDF and IDF fractions. In case of boiled BR the SDF fraction decreased by 75%, while that in boiled BW by 66%. The content of IDF fractions in boiled BR and BW dropped to a smaller level, i.e. by 47 and 17%, respectively. The ratio of SDF to IDF in raw BR was 1:1.8, while in BW groats it was 1:2.4 (Table 3). The SDF:IDF ratio changed as well, as it was 1:4 for boiled BR groats, whereas for boiled BW groats it was 1:6. According to GRIGELMO *et al.*. (1999), dietary fiber exhibits the best physiological properties at the ratio of soluble to insoluble fractions of 1.0-2.3.Thermal processing modifies the structure of dietary fiber. Extrusion cooking can increase the total dietary fiber content, while pressure cooking causes a reduction in the cellulose and hemicellulose content by degrading polysaccharides into simple sugars (ELLEUCH *et al.*, 2011).

3.2. Sorption of bile acids

The performed analyses showed sorption properties of the investigated groats to vary. Adsorption of bile acids was dependent on the type of acid and also its concentration. Among the analyzed acids DCA was bound to a highest degree. The acids at the concentration of 6 mM was bound to a higher degree than acid at the concentration of 1 mM (Table 4). Raw BR groats bound the highest amount of 6 mM DCA

Table 4 - Adsorption of bile acids by groats depending on type of bile acid and its concentration (g/100 g of product).

Kind of groats	Kind and concentration of bile acids			
	CA 1 mM	DCA 6 mM	1 mM	6 mM
Raw BR	17.64±0.15[b]	39.70±0.28[c]	35.97±0.25[d]	55.28±0.21[d]
Boiled BR	18.02±0.31[c]	27.50±0.15[b]	23.85±0.13[b]	32.78±0.17[a]
Raw BW	18.21±0.28[c]	43.43±0.28[d]	26.30±0.15[c]	50.98±0.28[c]
Boiled BW	12.02±0.25[a]	26.24±0.10[a]	18.70±0.10[a]	35.82±0.21[b]
Cellulose	3.16±0.05	4.18±0.08	2.19±0.03	2.75±0.06

[a-d] Values (means ± SD) in a column with different letters are significantly different at *P≤0.05*.
CA: cholic acid; DCA: deoxycholic acid; BR: barley groats; BW: buckwheat groats.

(55.28%), followed by 6 mM CA and 1 mM DCA, i.e. 39.7 and 35.97%, respectively, while the lowest binding capacity was observed in the case 1 mM CA (17.64%). Sorption of DCA, both at 1 and 6 mM, by BW groats was lower than BR groats, and amounted to 26.30 and 50.98%, respectively, while for CA it was higher and corresponded to 18.21% (1 mM) and 43.43% (6 mM). Groats after thermal processing showed much lower sorption in comparison to raw groats. Boiled BR and BW groats bound the greatest amount of 6 mM DCA (35.82% BW groats and 32.78% BR groats) and the lowest amount of 1 mM CA (18.02% BR groats, 12.02% BW groats). In comparison to raw BR groats sorption of 1 mM and 6 mM DCA was lower by 34 and 35%, respectively. Similarly, boiled BW groats showed lower sorption of both acids by 29 and 36%, respectively. In comparison to cellulose sorption of 6 mM DCA was by approx. 92% higher. According to CORNFINE *et al.* (2010), DZIEDZIC *et al.* (2012), KAHLON *et al.* (2007), KAHLON *et al.* (2008) the highest sorption capacity was found for lignin, while the lowest for cellulose. Considerable sorption capacities were also observed for pectins, whereas these capacities increase along with the degree of their esterification (BARBANA *et al.*, 2011; DONGOWSKI, 2007; KAHLON *et al.*, 2008). In contrast, hemicelluloses have a negative effect on the sorption capacity of bile acids (AMAROWICZ *et al.*, 1993). Higher sorption of 6 mM CA by raw BW groats in comparison to boiled BR groats may probably be connected with a much higher percentage of the lignin and hemicellulose fraction in dietary fibre (HU and YU, 2013). Chemical composition of fibre is not the only factor affecting the adsorption of bile acids. Other factors, such as porosity, surface area and adsorption force are also very significant (CORNFINE *et al.*, 2010; HUANG and DURAL, 1995; KAHLON *et al.*, 2007). Food processing may alter physico-chemical properties of dietary fiber, and thus also the capacity of fiber to absorb water and other organic components. Studies conducted so far indicate a hydrophobic character of secondary bile acid bound by dietary fiber. Authors of those studies showed that the rate of bile acid sorption decreases along with the increase in the number of hydroxyl groups in the steroid ring. This is connected with the varying numbers of hydroxyl groups it contains. The more hydroxyl groups, the lower the binding capacity of secondary bile acids (AMAROWICZ *et al.*, 1993; CORNFINE *et al.*, 2010; DONGOWSKI, 2007; HUANG and DURAL, 1995). Cholic acid is a trihydroxyl acid, i.e. one with lower sorption capacity, and it is a primary acid, while deoxycholic acid is a secondary acid, formed through metabolism of primary acids. Mutagenic properties have been attributed to secondary bile acids, thus their adsorption by dietary fiber seems to be of special importance from the nutritional point of view (BARRASA *et al.*, 2013).

4. CONCLUSIONS

The groats were characterized by different dietary fiber contents. Barley groats are characterised by higher dietary fiber content than buckwheat groats. The dominant fraction in all groats was hemicelluloses, while the smallest fraction was celluloses. The IDF level was higher than the SDF content. The groats were characterized by differing bile acid sorption capacity. Barley groats bound bile acids to a higher degree in comparison to buckwheat groats. Deoxycholic acid appeared to be more susceptible to binding than cholic acid. Acid at a higher concentration (6 mM/L) was better absorbed. Taking into consideration significant contents of dietary fiber in groats and sorption capacity in terms of bile acids, enrichment of everyday diet with these products is highly recommended, especially for people suffering from lipid disorders.

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PAPER

CHANGES IN POLYPHENOLIC COMPOSITION AND BIOACTIVITY OF BIJIA TEA DURING STORAGE

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ABSTRACT

The effect of storage on the composition and bioactivities of polyphenols in Bijia tea (*Camellia sinensis* [L.] Kuntze) was evaluated. Bijia tea samples were stored for 1, 3, or 6 years under identical conditions. As storage time increased, the polyphenol extraction yield, total polyphenol content, and amount of catechins decreased. Assays for polyphenol antioxidant activity and superoxide radical scavenging showed that both decreased with storage time. The inhibition ratio of Bijia tea polyphenols on histamine release from rat basophilic leukemia RBL-2H3 cells also decreased as storage time increased. This report provides the first description of Bijia tea polyphenols and their bioactivities as they change during storage.

- Keywords: *Camellia sinensis*, Bijia tea, storage time, catechin content, radical scavenging activity, histamine release inhibition -

INTRODUCTION

Second only to water, tea is the most ancient and popular beverage in the world for its refreshing and stimulating effects, as well as health benefits (KANWAR *et al.*, 2012; WU and WEI, 2002; de MEJLA *et al.*, 2009). To understand the mechanisms underlying these effects, the isolation and analysis of the active components in various teas have been conducted (ZUO *et al.*, 2002). In particular, there is great interest in the abundance of polyphenols such as catechins, flavonoids, anthocyanins, and lignans, which occupy 20-30% of the dry weight of tea leaves (KNAGGS, 2003; BOUDET, 2007). These are well known for numerous bioactivities (LEOPOLDINI *et al.*, 2006), and are considered the main contributors to the antioxidant, anti-inflammatory, and antimutagenic properties of tea (MOON and SHIBAMOTO, 2009; GALLEANO *et al.*, 2010; LAMBERT and ELIAS, 2010). Differences in the taste of various teas, e.g., ripened and aged Pu'er teas (stored for less or more than one year, respectively), are believed to be caused by variations in their polyphenol content (ZHANG *et al.*, 2011). Moreover, there are many reactions that can affect the polyphenol pattern during storage, including oxidation, polymerization, decomposition, and isomerization (ZHANG *et al.*, 2011; ZUO *et al.*, 2002).

Differences in methods of manufacture account for the many and varied commercial teas, including green, black, and oolong tea. An example of the latter is the Bijia tea, a traditional drink in Guangdong province, China. It is made with the tender leaves of *Camellia sinensis* (L.) Kuntze grown on the island of Bijia Mountain, Qingyuan. The manufacturing process of Bijia tea is significantly different from that of other oolong teas (DOU *et al.*, 2007). Simply, the leaves are cured in a wok, smoked by an open fire built from old pine tree branches, and then baked in a bamboo basket over a charcoal fire. The raw Bijia tea is stored in ventilated bags for 1 to 2 years. Finally, the leaves are steamed over boiling water for several minutes, and baked again. After all these steps, the Bijia tea is ready for sale.

Bijia tea has been famous for many years for its refreshing flavor and for its health-promoting features. It is often used as an expectorant to induce the production of fluids and soothe breathing passages. Aged Bijia tea develops a taste that is more complex, smooth, and mellow compared with that of newly ripened Bijia tea. Although there have been many studies of the chemical components, biological activities, and fermentation processes of various other teas (COOPER *et al.*, 2005; HOU *et al.*, 2009; QIN *et al.*, 2012; WANG *et al.*, 2008), there is little known about the chemical composition and in particular that of the phenolic compounds, of Bijia tea and how it is influenced by storage time.

The extraction, characterization, stability of pigments, and effects of storage time on the alkaloids of Bijia tea have been reported previously (WANG *et al.*, 2010; WU *et al.*, 2010). In the present study, an analysis of the polyphenols of Bijia tea and evaluation of the effects of storage time on their composition and bioactivity was carried out.

MATERIALS AND METHODS

Materials

Three Bijia teas were selected which had been stored at room temperature for 1 (B-I), 3 (B-II), or 6 years (B-III) in a cool, dry, and ventilated place. The leaves were harvested from the field on about Tomb Sweeping Day (Qingming Festival, ~March 8) at Bijia Mountain, Qingyuan, Guangdong, China.

Mast cells of the rat basophilic leukemia cell line RBL-2H3 were cultured at Shenzhen Key Laboratory of Microbial and Genetic Engineering, Shenzhen University, Shenzhen, China. All the biochemical reagents and chemicals were commercially available.

Extraction of polyphenols

Crude extracts were prepared by hot water reflux extraction at 90°C (1:50, w/w) for 30 min, and filtered (JIANG *et al.*, 2010). The combined filtrate for each experimental group was separated by column chromatography (packed with pretreated resin). To obtain polyphenols, the 80% ethanol/water eluent was concentrated under a vacuum at 45°C with a rotary evaporator (RV06-ML 1-B; IKA, Staufen, Germany), and then freeze-dried. All preparations were performed in triplicate. The extract yields were calculated as a percentage of the dry tea weight: extract yield, % = (weight of the extract / Bijia tea leaves, dry weight) × 100%.

Qualitative assay of polyphenols by chromogenic reactions

The presence of polyphenols in the extracts was proved by a qualitative chromogenic assay. On spot plates, 0.5 mL (5 mg/mL) of polyphenols of B-I, B-II, or B-III in water were added to 0.1 mL of ferric chloride, ferric chloride-potassium ferricyanide agent, or vanillin agent (WATERMAN and MOLE, 1994; FINE *et al.*, 2006; SUMBELE *et al.*, 2012). Color changes were observed and described after 2 min.

Estimation of total phenolic content

Total phenols of B-I, B-II, and B-III were quantified using Folin-Ciocalteau's reagents as described (ZHU *et al.*, 2002). Gallic acid was used as the standard reference and water was the control. All tests were performed in triplicate and expressed as mg/g of dry tea.

Quantitative analysis of polyphenols by high-performance liquid chromatography (HPLC)

The principal polyphenol components epicatechin (EC), epigallocatechin (EGC), and epigallocatechin gallate (EGCG; all from Sigma, USA) were quantitatively determined using HPLC (Agilent 1100, Agilent, USA) at 278 nm (Agilent PN 880952-702 column, SN USF0056126, 4.6 mm × 250 mm; flow rate 1.0 mL/min; mobile phase methanol [0.6% acetic acid/water]; gradient elution). All tests were performed in triplicate.

Analysis of free radical-scavenging activities

The ability of Bijia tea polyphenols to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals was assessed by the method described previously (ZHU *et al.*, 2002). Absorbance was measured at 517 nm (SpectraMax M2 Multi-Mode Microplate Reader, Molecular Devices, USA). Vitamin C was used as a positive control and methanol was the negative control. The antioxidant activity of polyphenols was defined as the clearance rate (CR_{DPPH}, %):

$$CR_{DPPH}\% = \frac{OD_{DPPH+MeOH} - \left(OD_{DPPH+Sample} - OD_{MeOH+Sample}\right)}{OD_{DPPH+MeOH}} \ 100\%$$

The effect of Bijia tea polyphenols on superoxide anion was detected with a superoxide anion detection kit (Superoxide Anion Free Radical Detection Kit, 50T, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and performed in accordance with the protocol of the manufacturer. All the above tests were performed in triplicate.

Histamine release analysis

The inhibitory effect of Bijia tea polyphenols on histamine release was analyzed as previously described (KIRITA *et al.*, 2010) with a slight modification. RBL-2H3 cells were seeded into 24-well plates (2×10^5 cells/mL, 1 mL/well) and cultured for 48 h. The cells were washed three times with Tyrode's buffer and treated with polyphenols (1.25, 2.5, 5, or 10 mg/mL) for 10 min after sensitization overnight with dinitrophenyl-specific IgE antibody (1 μg/mL). Then the cells were stimulated with antigen dinitrophenyl-bovine serum albumin for 90 min. After centrifugation, histamine release in the supernatants was determined with a mouse histamine enzyme-linked immunosorbent assay (ELISA) kit. All the above tests were performed in triplicate.

Cell treatment and assay of proliferation inhibition

The effect of Bijia tea polyphenols on the proliferation of RBL-2H3 cells was assessed by the method reported in Mosmann (1983) and in DENIZOT and LANG (1986) with minor modifications. The medium was removed and the cells were washed three times with PBS after incubation overnight in 96-well plates (2×10^5 cells/mL). RBL-2H3 cells were treated with tea polyphenols (0.625, 1.25, or 2.5 mg/mL) for 3 h. The medium was removed and the cells were washed again. Finally, the cells were incubated with a solution of 100 μL PBS and 20 μL MTS (3- [4,5-dimethylthiazol-2-yl] -5- [3-carboxymethoxyphenyl] -2- [4-sulfophenyl] -2H- tetrazolium) in each well for 4 h. The optical density was determined by measuring the absorbance at 490 nm using a microplate reader (GENios TecanAustria GmbH, Austria). Proliferation inhibition rates were quantified as a percentage relative to the blank. The test was performed in triplicate for each sample.

Statistical analyses

The mean value and standard deviation of analytes were calculated from the data obtained from the experiments. The statistical software package, SAS v9.1 (SAS Institute, Cary, NC, USA), was used for the analysis. The half-maximal inhibitory concentration (IC_{50}) was determined using Origin software (ver. 8.1).

RESULTS

Extraction of polyphenols

Bijia tea polyphenols were prepared using a combination of hot water reflux extraction and column chromatography. The reagents ferric chloride, ferric chloride-potassium ferricyanide, and vanillin were used to confirm that polyphenols were present in extracts from B-I, B-II, and B-III. The presence of polyphenols was indicated by brown, green, or red precipitates in response to these three reagents, respectively (Fig. 1).

The extraction yields of polyphenols from B-I, B-II, and B-III were 19.20, 17.56, and 15.67%, respectively, of the total weight of the original dry

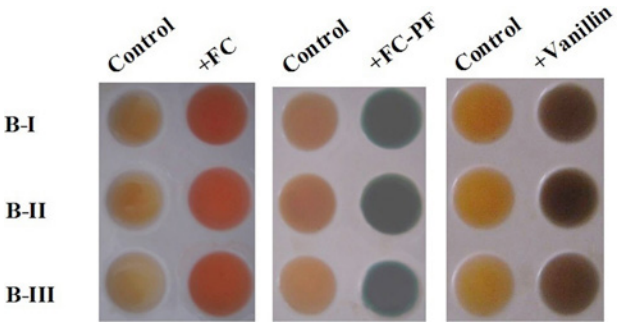


Fig. 1 - Chromogenic reactions of Bijia tea polyphenols. FC = ferric chloride agent, FC-PF = ferric chloride-potassium ferricyanide agent.

Table 1 - Extraction yields of polyphenol and total phenol contents of Bijia tea[a].

Bijia tea	Yield (%)	Total phenol content (mg/g)[b]
B-I	19.20±1.389	69.83±0.518
B-II	17.56±0.582	49.77±0.544
B-III	15.67±1.723	38.61±0.454

[a]Values are expressed as mean ± standard deviation (n = 3);
[b]Expressed as mg catechin/g dry tea.

Table 2 - Catechin content of Bijia teas[a].

Bijia tea	EGC[b]	EGCG[b]	EC[b]
B-I	15.96±1.080	5.167±0.119	1.047±0.061
B-II	8.177±1.003	1.981±0.050	Not detected
B-III	5.093±0.227	1.413±0.071	Not detected

[a]Values are expressed as mean ± standard deviation (n=3).
[b]Expressed as mg catechin/g dry tea.

weight tea sample (Table 1). Extraction yield decreased with time in storage. The total amount of phenols also decreased with storage time, as determined by spectrophotometry using gallic acid as a standard (Table 1).

Identification of main components of polyphenol extracts

Three main catechins, EC, EGC, and EGCG were quantified by HPLC (Table 2). It was found that EGC was the predominant catechin in Bijia tea polyphenols, followed by EGCG and EC. Similar to the polyphenol extraction yields and total phenol contents, amounts of these three catechins decreased remarkably with increased storage time. The EGC content of B-III was 31.9% that of B-I, and EGCG of B-III was 27.3% of B-I. Loss of phenols slowed down as storage time was prolonged, e.g. the total reduction of EGC content was greater from 1 year to 3 years (48.8%) than from 3 years to 6 years (37.7%).

Antioxidant activity of Bijia tea polyphenols

The antioxidative activity of Bijia tea polyphenols was evaluated by the DPPH assay. All samples exhibited a high ability to scavenge DPPH radicals after 30 min, and in a dose-dependent manner (Fig. 2). However, the antioxidative activity differed according to the storage time of the tea at each concentration. B-I polyphenols exhibited the highest antioxidative activity (IC_{50} at 0.0195 ± 0.0008 mg/mL), followed by B-II and B-III (IC_{50} at 0.0391 ± 0.0012 mg/mL and 0.0477 ± 0.0015 mg/mL, respectively).

The antioxidative effects were also determined by a superoxide radical scavenging assay. Bijia tea polyphenols had a moderate ability to scavenge superoxide radicals; the response was dose-dependent (Fig. 3). However, the differences between the observed potency of B-I, B-II, and B-III were not as significant as in the DPPH radical scavenging assay; the IC_{50}'s were 0.3789 ± 0.0034, 0.3788 ± 0.0025, and 0.4241 ± 0.0101 mg/mL, respectively.

Antiallergic activity analysis

The antiallergic effects of Bijia tea polyphenol extracts were measured in terms of the release of histamine from RBL-2H3 cells. All the polyphenols resulted to significantly inhibit the release of histamine in a dose-dependent manner, and the order of ability to inhibit histamine release was B-I > B-II > B-III. Specifically, the inhibition of histamine release due to polyphenols from B-I, B-II, and B-III was 32.39, 30.55, and 28.31%, respectively, at 5 mg/mL (Fig. 4).

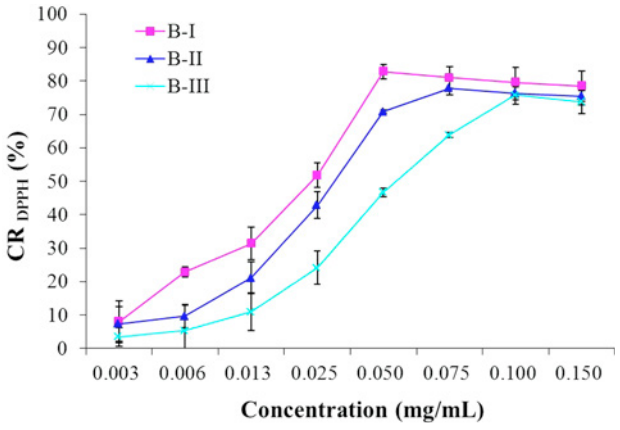


Fig. 2 - Antioxidant activity of Bijia tea polyphenols assessed by the DPPH radical-scavenging assay. CR_{DPPH} (%) = clearance rate of DPPH, expressed as mean ± standard deviation (n = 3).

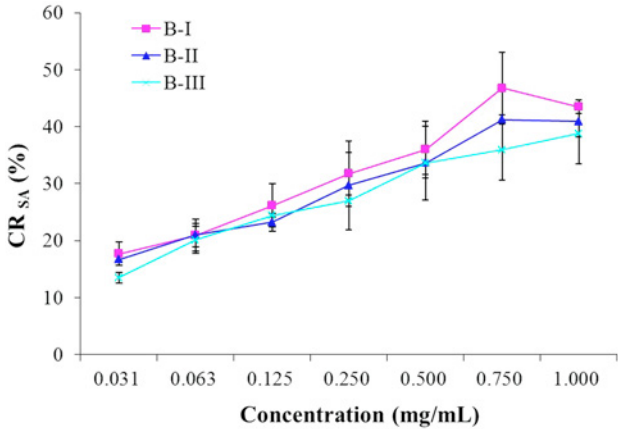


Fig. 3 - Antioxidant activity of Bijia tea polyphenols assessed by the superoxide radical. CR_{SA} (%) = clearance rate of superoxide anion, expressed as mean ± standard deviation (n = 3).

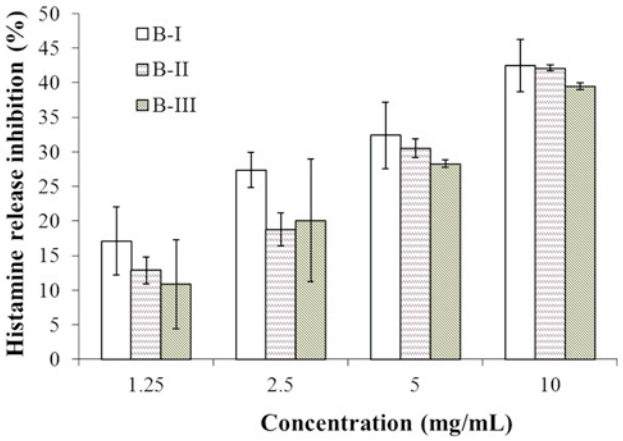


Fig. 4 - Inhibitory effects of Bijia tea polyphenols on histamine release in RBL-2H3 cells.
Data are expressed as mean ± standard deviation (n = 3).

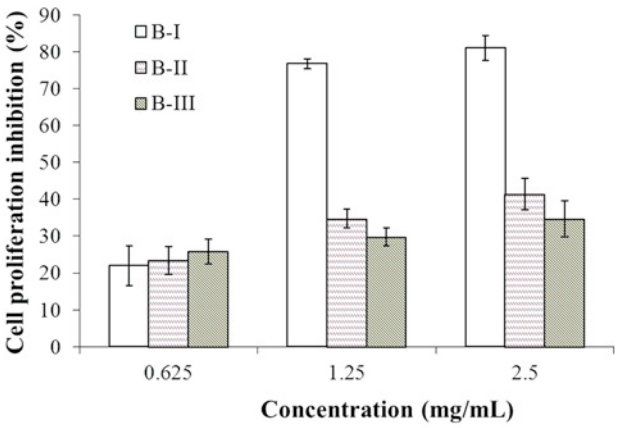


Fig. 5 - Inhibitory effects of Bijia tea polyphenols on the proliferation of RBL-2H3 cells.
Data are expressed as mean ± standard deviation (n = 3).

Furthermore, it was observed that proliferation of RBL-2H3 cells was also inhibited by Bijia tea polyphenol extracts in a dose-dependent manner (Fig. 5). At 1.25 mg/mL, the inhibiting effect of polyphenols from B-I (76.73%) was much higher than that of polyphenols from B-II (34.71%) and B-III (29.79%).

DISCUSSION

Interest in the health properties of tea and related scientific investigations has increased in recent decades (LIN *et al.*, 1998). Bijia tea has been a popular drink in southeastern China from ancient times. Interestingly, compared with ripened Bijia tea which is stored less than one year, aged Bijia is considered better tasting and is popularly believed to have greater health value. However, the components and related bioactivities of Bijia tea have been given very little scientific investigation. The present study focused on the primary polyphenolic components of Bijia tea and the effects of time in storage on their composition and bioactivities.

Tea extracts were first prepared by heat recirculating extraction and resin adsorption purification, from B-I, B-II, and B-III in order to achieve the highest recovery from the teas . The main components of the extracts were confirmed as polyphenols by traditional ferric chloride, ferric chloride-potassium ferricyanide, and vanillin colorable reactions (WATERMAN and MOLE, 1994; FINE *et al.*, 2006; SUMBELE *et al.*, 2012). Results showed that the polyphenol extraction yields and the total phenol contents of B-I, B-II, and B-III, which had been stored 1, 3, and 6 years respectively, decreased significantly following the same order.

To evaluate changes in Bijia tea polyphenol composition during storage, the relative contents of three main constituents, EGC, EGCG and EC, were determined. EGCG is the main catechin in green tea (ZUO *et al.*, 2002). However, in the present study the EGCG content in Bijia tea was much lower than that of EGC. This may be due to its high chemical reactivity, the effects of the half-fermentation process, or various oxidative decomposition reactions during storage (KANWAR *et al.*, 2012; ZUO *et al.*, 2002; BOUDET, 2007; ZHANG *et al.*, 2011).

Moreover, our analyses showed that ripened B-I contained higher amounts of EGC, EGCG, and EC than the aged B-II and B-III, and therefore it may be concluded that the level of catechins in Bijia tea was affected remarkably by the prolonged storage. Unlike Pu'er tea, in which the levels of catechins in aged Pu'er tea are higher than those of ripened (ZHANG *et al.*, 2011), the decrease of catechin content in Bijia tea with prolonged storage time may be due to the unique partial fermentation process, high activity of catechins, or the presence of microorganisms contained in Bijia tea. Because the phenolic hydroxyl groups in EGC, EGCG, and EC can be easily oxidized to quinones to form brown polymers (AKAGAWA and SUYAMA, 2001; AKAGAWA *et al.*, 2005); these catechins and their oxidized intermediates can react with amino acids, proteins, and other biomacromolecules to produce black polymers. This may also explain the different but more pleasant flavor of aged Bijia tea, and may also be the reason that B-III is darker than B-II, which is darker than B-I.

Increasing research findings suggest that the antioxidant properties and radical scavenging of tea polyphenols, which are highly correlated with molecular weight and the number of phenolic hydroxyl groups, are responsible for the beneficial effects of tea in different chronic diseases (HIGDON and FREI, 2003; HALLIWELL, 1995; LILLIAN, 1995). Here, the antioxidation of Bijia tea polyphenols was proved by means of the DPPH and superoxide radical scavenging assays. Bijia

tea polyphenols exhibited a remarkable DPPH scavenging effect and powerful superoxide radical scavenging ability. The observed rank order was consistently B-I > B-II > B-III, which appears related to the decrease in the content of catechins in Bijia tea polyphenols and the decrease in phenolic hydroxyls during storage. Furthermore, this order agrees with that of the polyphenol extraction yields and total polyphenol content from B-I, B-II, and B-III.

Allergies are caused by excessive immune activity. Up to 30% of the worldwide population is affected, and the incidence rate is increasing (LEUNG *et al.*, 2012). In the laboratory, inhibition of histamine release from mast cells is the principle means of evaluating the allergenic properties of chemicals (GALLI *et al.*, 1999; KINET, 1999; KAWAKAMI and GALLI, 2002). In the present study, the antiallergic effects of Bijia tea polyphenols *in vitro* was investigated and it was found that Bijia tea polyphenols inhibited the histamine release from RBL-2H3 cells induced by IgE, in a dose-dependent manner. Moreover, Bijia tea polyphenols inhibited the proliferation of RBL-2H3 cells, although the inhibiting potency of B-I was much stronger than that of B-II or B-III at 1.25 mg/mL. These results prove that the antiallergic properties of Bijia tea decreased as storage time increased. That may be due to the sharp decrease in EGC and EGCG in B-II and B-III because of the longer storage time. Our findings regarding the antiallergic function of tea polyphenols is supported by other reports (MAEDA-YAMAMOTO *et al.*, 2012; SANO *et al.*, 1999; SUZUKI *et al.*, 2000; FUJIMURA *et al.*, 2002; MAEDA-YAMAMOTO *et al.*, 1998).

CONCLUSIONS

Extract yields, total phenols, the predominant catechins, and the antioxidative and antiallergic effects of Bijia tea all decreased remarkably with time in storage. Further studies on the newly formed components and health benefits of aged Bijia tea are warranted.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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SHORT COMMUNICATION

EFFECTS OF GASEOUS OZONE ON FOOD-BORNE PATHOGENS

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ABSTRACT

The aim of this study was to evaluate the effect of gaseous ozone on *Staphylococcus aureus* (*S. aureus*) and *Listeria monocytogenes* (*L. monocytogenes*) strains with regard to biofilm production and survival. The experiment was conducted with six *S. aureus* strains and four *L. monocytogenes* strains. After exposure to ozone, the biofilm-forming abilities of bacteria were assessed. The treatment caused a mean reduction of 0.82 and 1.39 log units for *S. aureus* and *L. monocytogenes*, respectively. Although the effect of ozone gas on cell survival was minimal, biofilm production was affected. Much effort should be invested in examining the effects of gaseous ozone treatment on bacterial biofilm formation.

- Keywords: biofilm, food-borne pathogens, gaseous ozone -

INTRODUCTION

It is known that the most common ways for bacteria to live is adhering onto surfaces and forming organized communities named biofilms. Cleaning and disinfection operations play a key role in ensuring the safety and quality of food products. Almost 45-70% of the total water used within the food industry is for sanitation operations. Initiatives at national and international levels involve the reduction of water consumption in food industry. With regard to this, ozone (O_3) may offer in the food industry an alternative or complementary cleaning and sanitizing agent (KIM *et al.*, 1999; GUZEL-SEYDIM *et al.*, 2004). The use of O_3 may save water in comparison to other biocides, as it does not leave residues; therefore it does not require a final rinse to remove any residual disinfectant that might remain (PASCUAL *et al.*, 2007; ÖLMEZ and KRETZSCHMAR, 2010). The antimicrobial action of O_3 has been studied and documented on a wide variety of organisms (RESTAINO *et al.*, 1995). O_3 can be applied in either aqueous (RESTAINO *et al.*, 1995) or gaseous phase (KIM *et al.*, 2003). Although in the last year, the reduction of bacteria by O_3 in aqueous phase has been studied extensively, minimal information is currently available about the inactivation of food-borne pathogens with gaseous O_3. In addition, studies addressing the action of O_3 on biofilm-forming ability of microorganisms are scarce. Therefore, the aim of the present study was to evaluate the effect of gaseous O_3 on strains of *Staphylococcus aureus* (*S. aureus*) and *Listeria monocytogenes* (*L. monocytogenes*) with regard to biofilm production and survival.

MATERIALS AND METHODS

Bacterial strains

Two representative food-borne pathogens, such as *S. aureus* and *L. monocytogenes*, were used in this study. The experiment was conducted with ten strains, i.e. 6 *S. aureus* strains (n. 1 ATCC 29213; n. 5 strains isolated from poultry meat: A, B, C, D, E) and 4 *L. monocytogenes* strains (n.1 ATCC 19111; n. 3 strains isolated from poultry meat: a, b, c) from the bacterial culture collection of the Department of Food Science - University of Parma. Prior to the conduction of experiments, all strains were activated by culturing in 10 mL of Tryptic Soy Broth (TSB-Oxoid S.p.A., Milan, Italy) twice at 37°C for 24 h and serial decimal dilutions in TSB were prepared.

Ozonation assays

Ozonation assays were carried out in a glass chamber (200 L volume) under complete mixing conditions using a continuous flow of O_3 at a temperature of 20° ± 0.5°C. The relative humidity (RH) inside the chamber was at 70 ± 3%. O_3 gas was generated using a corona discharge ozone generator (ASLAN S.r.l.). The O_3 concentration in the chamber, during the treatment time (20 min), was measured using an O_3 analyzer with a measuring range from 0.1 ppm to up 10 ppm O_3. Appropriate dilution in TSB of each strain was inoculated in Tryptic Soy Agar (TSA-Oxoid S.p.A., Milan, Italy) plates. During the experimental work, O_3 was fed into the upper chamber until the concentration reached: 2.5 ppm. The TSA plates inoculated with *S. aureus* and *L. monocytogenes* strains with O_3 treated were incubated at 37°C for 24 h and the inoculated TSA plates not submitted to ozonation were control tests. All plate count data obtained were transformed into $\log_{10}$ values. In all cases triplicate samples were used for each assayed condition.

Biofilm formation

In this study, a previously described methods was used (DI BONAVENTURA *et al.*, 2008). After the treatment with gaseous O_3 at 2.5 ppm for 20 min, cultures of *S. aureus* and *L. monocytogenes* were recovered from overnight TSA-growth and were transferred in TSB. All strains were tested for biofilm formation and the results were compared with the not treated strains. In particular, AISI 304 stainless steel chips (530 mm^2) were used as substrata for bacteria biofilm formation at 12°C. All results were expressed by calculating a "Biofilm Production Index" (BPI) as follows: $BPI = [OD_{meanbiofilm} \text{ surface } (mm^2)^{-1}] \times 1{,}000$. All experiments were carried out in triplicate and repeated in two independent sets of experiments.

RESULTS AND CONCLUSIONS

O_3 treatment caused a reduction of 0.82 and 1.39 log units (mean reduction) on *S. aureus* and *L. monocytogenes*, respectively (Table 1). As their biofilm-forming abililies are concerned, the results expressed in BPI are shown in Fig. 1. All the

Table 1 - Log reduction of *S. aureus* and *L. monocytogenes* strains after 20 min of exposure to O3 at 2.5ppm.

Strains	Initial population (Log CFU/mL)†	Population after treatment (Log CFU/mL)†	Log reduction (Log CFU/mL)
S.a ATCC	8.13±0.08	6.61±0.55	1.52
S.a A	8.41±0.10	7.50±0.17	0.91
S.a B	8.07±0.03	7.50±0.14	0.57
S.a C	8.32±0.02	7.52±0.14	0.80
S.a D	7.70±0.06	7.34±0.09	0.36
L.m ATCC	8.62±0.06	7.23±0.20	1.39
L.m a	9.24±0.07	7.68±0.21	1.56
L.m b	8.26±0.07	7.23±0.08	1.03
L.m c	8.36±0.02	6.77±0.30	1.59

†Values are means of three determinations ± standard deviation.

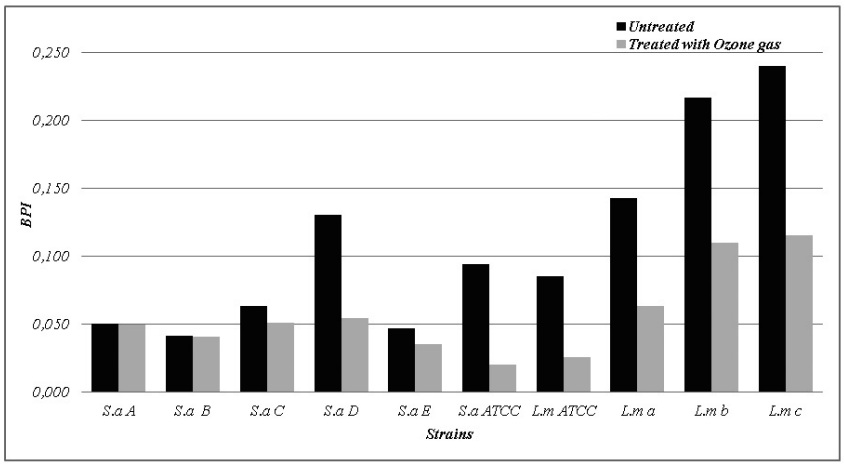


Fig. 1 - Biofilm formation by *S. aureus* and *L. monocytogenes* strains on stainless steel incubated at 12°C. Biofilm intensity is expressed as Biofilm Production Index (BPI).

strains tested were able to adhere and form biofilm on stainless steel at 12°C. *L. monocytogenes* strains formed higher average amount of biofilm than *S. aureus* strains on stainless steel at 12°C. Among the *S. aureus* strains the effect of ozone gas was minimal on *S. aureus* D (0.36 log reduction). In spite of this, the biofilm-forming ability of *S. aureus* D was affected and the treated strain showed a lower BPI (0.055) compared to the untreated strain (0.13). This study revealed that the effect of O_3 gas was minimal on cell survival. These findings are in contrast to the study of ROBBINS *et al.* (2005). Anyway, the different design of the two studies does not really allow a clear comparison of the results. In this study, in accordance with RESTAINO *et al.* (1995), the effect of O_3 gas differed between *S. aureus* and *L. monocytogenes* strains and *L. monocytogenes* was more sensitive to O_3 than *S. aureus* . Moreover, some strain-specific variation was observed for both of *S. aureus* and *L. monocytogenes*. Our results suggest that *S. aureus* and *L. monocytogenes* strains are able to adhere and form biofilm on the tested surface at low temperature (12°C) used in the food industry during processing. Although we tested a limited number of strains, *L. monocytogenes* formed higher average amount of biofilm than *S.aureus* on stainless steel at 12°C. As for the usage of O_3 in gaseous form, few studies on its efficiency and effectiveness in disinfecting stainless steel surfaces have been reported in the literature. Additionally, studies addressing the effectiveness of gaseous O_3 on microorganisms and how that exposure also affects their biofilm-forming abilities are scarce. Summarizing the above finding, it only can be concluded that the dosage of O_3 used (2.5 ppm) slightly reduced the population of the bacterial culture. Although the effect of ozone gas was minimal on cell survival, biofilm production was affected. The slight reduction in biofilm formation may reflect the number of dead cells in the inocula in the bacterial population treated. In conclusion, further studies are needed to confirm these initial findings.

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