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PAPER

VOLATILE PROFILE AND SENSORY CHARACTERISTICS OF CREAMS USED AS FILLERS OF BAKERY PRODUCTS: HOME MADE EGG CREAMS VS INDUSTRIAL CREAMS

PROFILO IN COMPOSTI VOLATILI E CARATTERISTICHE SENSORIALI DI CREME UTILIZZATE COME FARCITURA DI PRODOTTI DA FORNO: CONFRONTO TRA CREME A BASE D'UOVO FATTE IN CASA E CREME INDUSTRIALI

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ABSTRACT

Volatile profiles of creams used as fillers of bakery products including home-made egg yolk creams and industrial creams were compared. A headspace solid phase microextraction method coupled with GC-MS was used. A total of 82 volatile compounds were identified. The volatile groups were aliphatic

RIASSUNTO

Sono stati confrontati i profili in composti volatili di creme utilizzate come farciture di prodotti da forno quali creme a base di tuorlo d'uovo fatte in casa e creme industriali. È stato utilizzato un metodo di microestrazione in fase solida accoppiato con la GC-MS. Sono stati identificati un totale di 82 composti volatili.

- Key words: bakery products, cream, egg yolk, sensory analysis, SPME, volatiles -

and alicyclic hydrocarbons (18), aldehydes (10), ketones (10), aromatic hydrocarbons (19), furans (6), alcohols (12), esters (3) and acids (4). The volatile profiles of home-made creams and industrial creams differed significantly. Chemometric analysis of the volatile data was successfully used to predict cream origin. The descriptive flavour profile of the different types of creams was elaborated. Pearson correlation was applied to flavour attributes and volatile compounds, but, few correlations were found, because several effects such as the presence of fat, starch and sweetness influenced aroma release.

I gruppi volatili erano idrocarburi alifatici ed aliciclici (18), aldeidi (10), chetoni (10), idrocarburi aromatici (19), furani (6), alcoli (12), esteri (3) e acidi (4). I profili in composti volatili delle creme fatte in casa e delle creme industriali differivano significativamente. L'analisi chemiometrica dei dati sui volatili è stata utilizzata con successo per predire l'origine della crema. È stato elaborato il profilo descrittivo aromatico delle differenti tipologie di crema. La correlazione di Pearson è stata applicata agli attributi sensoriali ed ai composti volatili, ma sono state trovate poche correlazioni perché numerosi effetti quali la presenza di grasso, amido e la dolcezza influenzavano il rilascio dell'aroma.

INTRODUCTION

Fresh raw egg yolk has a bland odour. However, upon heating it develops a fairly characteristic pleasant aroma. Egg yolk contains proteins and lipids in large quantities. Consequently, lipid oxidation and Maillard reactions are the most important pathways leading to egg yolk odorants. Egg yolk is a common ingredient in cooking and baking.

Home-made egg creams used as fillers for bakery products (ECBP) are widely used. Traditionally, these creams are prepared with egg yolk. Two different methods have been used; in one, sugar and water are heated at 110°C for 15 min and egg yolk, flour and vanillin are added after cooling to 60°C (ECBP1). In the other method, milk, sugar and flour to which egg yolk and vanillin have been added (ECBP2) are carefully heated (<100°C). Presently, industrial powders in which egg yolk is replaced by egg aromatising compounds and colour compounds are used to prepare creams that are used as fillers of bakery products (ICBP).

The flavour of traditional home-made ECBP is the result of the aroma compounds produced by thermal reactions during heating of egg yolk, sugar, flour and other ingredients. Previous studies of the aromatic compounds in baked products have mainly focused on bread and bread-making steps (GALEY *et al.,* 1994; GOBBETTI *et al.*, 1995; HANSEN and HANSEN, 1994; KOMAITIS and AGGELOUSIS, 1993; SCHIEBERLE and GROSCH, 1994; ZEHENTBAUER and GROSCH, 1998a,b), on cookies (PROST *et al.*, 1993) and on sponge cake (POZO-BAYÓN *et al.,* 2007). In addition, several authors have analysed the volatile compounds of heated egg and egg yolk (HSU and CHEN, 1981; LORENZ and MAGA, 2006) MACLEOD and CAVE, 1975, 1976; RAYER *et al.*, 1980; UMANO *et al.*, 1990) and the volatile compounds that contribute to the aroma. The amounts of volatile compounds in heated egg yolk have been identified (CERNY and GUNTZ,

2004). To our knowledge, no study on the volatiles present in ECBP and ICBP has been published.

Three methods for extracting volatiles from eggs and egg products have been described, including, simultaneous steam distillation-extraction (SDE) with diethyl ether as the organic solvent according to the Likens-Nickerson method (UMANO *et al.,* 1990) and headspace (HS) methods, such as, dynamic headspace (Purge and trap) and static headspace (solid phase microextraction –SPME) using polydimethylsiloxane/divinilbenzene - PDMS/DVB fibre (ADAMIEC *et al.,* 2002).

Headspace solid-phase microextraction (HS-SPME) is a simple, fast, sensitive and solvent-free extraction technique that allows the extraction and concentration steps to be performed simultaneously (PINHO *et al.,* 2003, 2004, 2006). SPME fibres with a great range of selectivity for evaluating volatile compounds are available on the market. The CAR-PDMS 75 µm phase is a porous material (micro-, meso-, and macropores from 6 to 50 Å) that increases retention capacity due to the mutually potentiating effect of adsorption to and distribution within the stationary phase. Thus, a complete profile of volatile compounds in food can be obtained.

As HS-SPME is an equilibrium-based technique, extraction temperature and time greatly influence the amount of analyte extracted. The goal however, is not to perform an exhaustive extraction, but an extraction in pre-equilibrium conditions in order to minimize extraction time while maximizing extraction efficiency. The procedures need to be optimized by examining several parameters, namely, equilibrium time, extraction time and temperature. Reproducibility should also be evaluated.

The aim of the present work was to study the volatile fraction of home-made egg creams used as fillers for bakery products (ECBPs) and compare the results with those of the volatile fraction of industrial creams used for the same propose (ICBPs). Chemometric analysis of the volatile data was also used to predict cream origin. The descriptive flavour profile of the different types of creams was elaborated and correlated with chemical data.

MATERIAL AND METHODS

Sampling

Three batches of each home-made egg cream used as fillers for bakery products were included in the experimental design to cover the variation in the preparation process. Three batches of home-made egg cream 1(ECBP 1) were prepared with 100 g of sugar and 50 mL of water, heated at 110°C for 15 min, cooled to 60° and 45 g of egg yolk, 10 g of flour and 0.2 g of vanillin were added, to obtain 110 g of ECBP 1 (viscosity: 170000 cps). Three batches of home-made egg cream 2 (ECBP 2) were prepared by carefully heating (to 100°C) 400 mL of milk, 100 g of sugar and 20 g of flour to which 45 g of egg yolk and 0.5 g of vanillin were added to obtain 375 g of ECBP 2 (viscosity: 140000 cps). The viscosity properties of the egg creams were evaluated using a Brookfield viscometer (Model DVIII, Stoughton, MA, USA). The spindle speed was set at 1.5 rpm and a n. 3 spindle was used. The experiment was run at room temperature (25±1°C). Viscosity was measured for 1 min. Egg creams were analysed for volatiles and flavour on the day of preparation.

Three different lots of the same brand of an industrial cream used as a filler for bakery products with a known label were analysed (ICBP 0). It was composed of sugar, modified starch, skimmed powdered milk, hydrogenated vegetable fat, thickener (E401), acidity regulator (E 450), salt, colorants (E102 and E124), preservatives (E200 and E202) and aro-

mas. These creams were prepared by mixing 100 g of powder and 100 g sugar; 300 g of cold water were added slowly and mixed for 3 min at high speed.

Four brands of industrial creams (ICBP) collected from "bolas de Berlim" bakery products were randomly purchased and numbered from ICBP 1 to ICBP 4 and analysed on the day they were purchased. Three lots of each brand were analysed. Since there was no label describing the composition of these ICBP, the major components were evaluated in the laboratory.

Extraction of volatile compounds

The creams were homogenised using a spatula and 3 g samples were taken and placed in a 15 mL vial subsequently sealed with PTFE-silicone septa (Supelco, Bellefonte, PA, USA). Carboxen/PDMS fibre (Supelco, Bellefonte, PA) was used to extract the volatile cream compounds. Each cream was analysed in quadruplicate. Sample vial equilibration was performed at 40°C for 30 min; the CAR/PDMS fibre was then exposured to the headspace above the sample for 30 min followed by thermal desorption of the adsorbed substances in the injector port GC-MS analysis (PINHO *et al.*, 2003, 2006).

GC-MS analysis

The analyses were performed using a Hewlett-Packard (HP), model 6890 (Hewlett-Packard Company, Palo Alto, CA, U.S.A.), GC fitted with a splitless injector suitable for SPME analysis and Agilent 5973 mass spectrometer (MS) detector (Agilent Technologies, Santa Clara, U.S.A.). Helium was used as the carrier gas with a flow rate of 1 mL/min. The components were separated on a SPB-5 capillary column 60 m x 0.32 mm x 1.0 μm-film thickness, (Supelco, Bellefonte, PA, U.S.A.). The oven temperature program was 5 min at 40°C and then 3°C min^{-1} to 200°C for 5 min. The injector temperature was 290°C (PINHO *et al.*, 2004). Detection was by mass spectrometry on the total ion current obtained by electron impact at 70 eV. The constituents were identified by comparing the experimental spectra with spectra from Nist 98 data bank (NIST/EPA/NISH Mass Spectral Library, version 1.6, U.S.A.), using a match factor higher than 97%. When possible, the identity of the volatile compound was also confirmed using an authentic standard. Propanal, hexanal, 3-methylbutanal, nonanal, decanal, 2-methylpropan-1-ol, 3-methylbutan-1-ol, octanoic acid and hexan-1-ol (purity: 99%) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); acetic acid, acetone, butan-2-one, ethanol, 2-methylpropan-1-ol, toluene, 1,4-xilene, phenol, 4-hydroxy-3-methoxy-benzaldehyde, benzaldehyde and benzoic acid were from Merck (Darmstadt, Germany). Based on the peak resolution, their areas were either calculated from the total ion current or estimated from the integrations performed on selected ions. The resulting peak areas were expressed in arbitrary units of area. Reproducibility was assessed as the relative standard deviation (RSD) of a 6-fold analysis.

Evaluation of nutritional composition of ICBP

The total protein content, ash and fat were determined according to AOAC methods (2000). Quantification of carbohydrates was performed by HPLC with an evaporative light scattering detector (NOGUEIRA *et al.*, 2005). The chromatographic analyses were carried out in an analytical HPLC unit (Jasco, Tokyo, Japan), equipped with a low pressure quaternary pump (PU-1580), an evaporative light scattering detector (LSD, Sedex 75, France) and a type 7125 Rheodyneinjector with a 10 μL loop. A Borwin Controller Software (JMBS Developments, Grenoble, France) was also used. The chro-

matographic separation was achieved using a Spherisorb NH_2, 5μ chromatographic column (250mm×4.6mm i.d.) and gradient elution with solvent A (acetonitrile)/solvent B (water). Carbohydrates were eluted by increasing the proportion of solvent B from 19 to 25% over 40 min: 0-19 min, 19% B; 20-40 min, 25% B. The flow-rate was 1 mL/min. The temperature of the heated drift tube was 45°C, the gas pressure was 3.0 bar and gain 5.

Sensory analysis

Fourteen panellists (Master Porto University students) were recruited to participate in a descriptive flavour analysis. Eleven flavour attributes, arranged in non-structural scales, were used to describe the creams under study. Using different egg creams and industrial creams the panellists were trained, in six one-hour sessions for term optimization and calibration for accuracy in interpretation and repeatability.

Training sessions

In Session 1, panellists performed triangle tests using modified egg creams, to determine if there was a perceptible sensory difference between samples of two egg creams made with different amounts of ingredients (sugar, eggs, flour and vanillin).

In Session 2, panellists tasted creams with specific, highlighted flavour attributes. Panellists were invited to generate terms to describe personal observations. In Session 3, redundant descriptive terms were removed and samples exhibiting specific attributes to be included on the ballots were tasted. Session 4 was designed to establish ballot anchors in which all attributes and their synonyms were arranged in an unstructured scale (7 points). To assist panellists, terms were used to describe each attribute at low intensity (1) and high intensity (7). In Session 5, the ballots were tested by panellists in individual booths with unknown representative samples. The collected data were analyzed by analysis of variance (ANOVA) (SPSS), and panellist deviations were assessed to determine where additional training was needed. During Session 6, tasting results and panellist deviations were discussed and specific terms and anchors were clarified.

Evaluation Sessions

For the experimental testing all samples, including, egg creams (ECBP 1, ECBP 2), and industrial creams (ICBP 0 to ICBP 4), were labelled with random three-digit codes. In each session, panellists received a maximum of seven samples to evaluate. The experimental samples were served to panellists in random order, two sessions were performed.

Statistics

Exploration of data, descriptive statistics, t-test and ANOVA with pairwise comparisons of mean values using Tukey's test, Pearson correlation and discriminant analysis were performed with SPSS for Windows version 13 (SPSS, Chicago, IL, USA).

RESULTS AND DISCUSSION

Volatile compounds identified in the cream

An equilibrium time and extraction time of 30 min at 40°C were chosen as a good compromise between analysis time and sensitivity. Reproducibility was satisfactory; the RSD values were less than 10%. A total of 82 volatile compounds were tentatively identified in ECBP1, ECBP 2, ICBP 0, ICBP 1, ICBP 2, ICBP 3 and ICBP 4 (Table 1). An example of a chromatogram of an industrial cream is

Table 1 - Volatile fraction of classes of compounds in creams used as fillers for products identified by HS-SPME-GC-MS.

	Compound	RT	ECBP 1	ECBP 2	ICBP 0	ICBP 1	ICBP 2	ICBP 3	ICBP 4
		Results expressed as arbitrary units of area x 10^3							
	Aldehydes								
1	Isobutanal	7.347				2079		1091	
2	*propanal	8.580			10141				42805
3	(E)-but-2-enal	12.449				1017	1998		1264
4	*3-methylbutanal	12.680	3786		68747	6927	11212	22761	18508
5	*pentanal	14.923		8154					
6	hexanal	21.200	27818	13140	12487	12610	34669	18084	31380
7	*heptanal	27.569	4201		2274				
8	*octanal	33.669							12831
9	nonanal	39.360	1997			1370	2183	1534	1633
110	*decanal	44.671	7032		3230				63723
	Ketones								
11	*acetone	6.884			69450				
12	butane-2,3-dione	9.631				1671		1128	
13	*butan-2-one	9.976		19450					
14	3-hydroxybutan-2-one	15.549				17231	10612	33512	16412
15	heptan-2-one	26.784						14119	1011
16	*cyclopentane-1,2-dione	28.955					6625		
17	*5-methyl-1H-pyrimidine-2,4-dione	37.558					40363		
18	3,5-dihydroxy-2-methyl-5,6-dihydropyran-4-one	41.764					1386		
19	*4-hydroxy-4-methyl-2,6-ditert-butyl-cyclohexa-2,5-dien-1-one	57.297	8976			15402	14390		
20	2,6-ditert-butylcyclohexa-2,5-diene-1,4-dione	57.538				1062			
	Aromatics								
21	toluene	19.408	4286						8359
22	*1,4-xylene	25.937	3920	6765					
23	*N-methylidenehydroxylamine-methoxyphenyl	26.833	6166	7482	11625		10618		10817
24	benzaldehyde	31.547	1645	1611					1684
25	phenol	32.128			1775				
26	1,2,4-trimethylbenzene	33.583						1221	
27	1-methyl-2-propan-2-yl-benzene	35.338							
28	*4-hydroxy-3-methoxy-benzaldehyde	54.369	2471	3124	27271	68522	74221	26510	2312
29	4-methyl-2,6-ditert-butyl-phenol	59.22	1370			9125			
30	5-phenyldecane	60.061	1498			1387			
31	*4-phenyldecane	60.440	28017			25412			
32	*3-phenyldecane	61.260	28676			21449			
33	*2-phenyldecane	62.71				6664			
34	*6-phenyldodecane	63.563	8409			10392			
35	6-phenylundecane	63.817					1856		
36	5-phenylundecane	63.951	1983			1334	942		
37	*4-phenylundecane	64.382	46959		5166	56525	12270		5166
38	3-phenylundecane	65.255	34239			37184			
39	*2-phenylundecane	66.741	13418			16296			

	Compound	RT	ECBP 1	ECBP 2	ICBP 0	ICBP 1	ICBP 2	ICBP 3	ICBP 4
Hydrocarbons									
40	*cyclopenta-1,3-diene	7.893		32905					
41	*2-methylpentane	8.947		35825	21401				21401
42	*hexane	10.137			13307		31577		133075
43	*hept-1-ene	14.652		9906					
44	1,1-diethoxyethane	16.903				1949			
45	*pent-1-ene	19.118		5399					
46	*2,6-dimethyloctadecane	33.494		8282					
47	1-methyl-4-prop-1-en-2-yl-cyclohexene	35.639	1224					1374	1211
48	heptadecane	37.175				1189			
49	*2,2,3-trimethylnonane	38.454		6679					
50	*dodecane	39.160		31045	2907				
51	pentadecane	48.779				1740			
52	*tridecane	49.174	4129						
53	*icosane	50.093	7233						
54	*tetradec-1-ene	53.795	2796			6782			
55	*nonedecane	60.741	4957						
56	hexadecane	62.198				1789			
57	2,6,10,14-tetramethylpentadecane	66.355	1273			1050			1101
Furans									
58	*2-methylfuran	10.36			16719				
59	*furan-2-carbaldehyde	23.37		8207	51318		68624		
60	*2-furylmethanol	24.59			2254		24081		
61	*5-methylfuran-2-carbaldehyde	31.441	9255		90550				
62	*4,5-Dimethyl-2-formylfuran	37.85			36788		32203		
63	* 5-(hydroxymethyl)furan-2-carbaldehyde	44.34					15311		
Alcohols									
64	*ethanol	6.204			71312				
65	*2-methylpropan-1-ol	11.311	11722		67385			48921	6710
66	butan-1-ol	13.091			2311			1838	3240
67	3-methylbutan-1-ol	17.129	2931			1810	1281	3310	1021
68	2-methylbutan-1-ol	17.361				1011	1061		1941
69	*pentan-1-ol	19.114							15776
70	*butane-2,3-diol	19.570			4587				14748
71	*hexan-1-ol	25.453			19549	17392	17392	11042	
72	*2-butoxyethanol	27.853				8661	8661		57298
73	*2-(2-ethoxyethoxy) ethanol	33.645			5321				
74	*2-ethylhexan-1-ol	35.088	3387						
75	2-phenylethanol	40.215						1125	1016
Esters									
76	ethyl acetate	9.407			1666	1721		2810	1666
77	phenyl benzoate	16.809							1119
78	*butyl acetate	22.071	5677						
Acids									
79	Acetic acid	9.561	1120		4261		2413	2310	2111
80	2E,4E)-hexa-2,4-dienoic acid	37.475			1321			1282	
81	*benzoic acid	41.96					6053		
82	*octanoic acid	42.27			61571				

* ANOVA analyses indicate significant differences between samples (expressed as arbitrary units of área).
RT - retention time.

presented in Fig.1. The chemical families of compounds agree with those reported by other authors (GROSCH, 1987). The classes of volatiles detected were aliphatic and alicyclic hydrocarbons (18), aldehydes (10), ketones (10), aromatic hydrocarbons (19), furans (6), alcohols (12), esters (3) and acids (4).

ECBPs and ICBPs presented different qualitative and quantitative volatile profiles as can be observed in Table 1 and Fig. 2. The most abundant classes of compounds in ECBP were aromatics, aldehydes and hydrocarbons and, significant differences ($p<0.05$) were observed for those classes of compounds for ECBP 1 and ECBP 2. Fig. 2 presents the distribution of the compound classes in the cream samples.

According to the literature, aldehydes are the major volatile components in cooked egg yolk (UMANO *et al.,* 1990). These volatile compounds are described as egg odourants resulting from lipid oxidation. It is well known that lipid oxidation is an important factor for the formation of aroma compounds. Egg yolk lipids are rich in oleic and linoleic acids and contain a considerable amount of arachidonic acid. Certain amino acids found in egg yolk, valine, alanine, leucine, methionine and phenylalanine, also play a role in the formation of aldehydes (ELMORE *et al.,* 2005; HUANG *et al.,* 2004) Five aldehydes, including 3-methylbutanal, hexanal, heptanal, nonanal and decanal were detected in ECBP1, while ECBP2 only had pentanal and hexanal (Table 1). It should be pointed out that ECBP1 has more concentrated egg yolk (45 g of egg yolk in 110 g of ECBP 1, in comparison with 45 g of egg yolk enable in 375 g of ECBP 2), thus, it is understandable that ECBP 1 had higher peak area values for aldehydes (Table 1).

Only one ketone was detected in each ECBP, 4-hydroxy-4-methyl-2,6-ditert-butyl-cyclohexa-2,5-dien-1-one and butan-2-one (Table 1). The last one was also detected in cooked egg and egg yolk heated to 50°C (STEIN and NARANG, 1990). Numerous ketones have been reported to be products of carbohydrate caramelization and pyrolysis (UMANO *et al.,* 1990), indicating that in the preparation of the ECBPs sugar heating in the preparation was obviously mild.

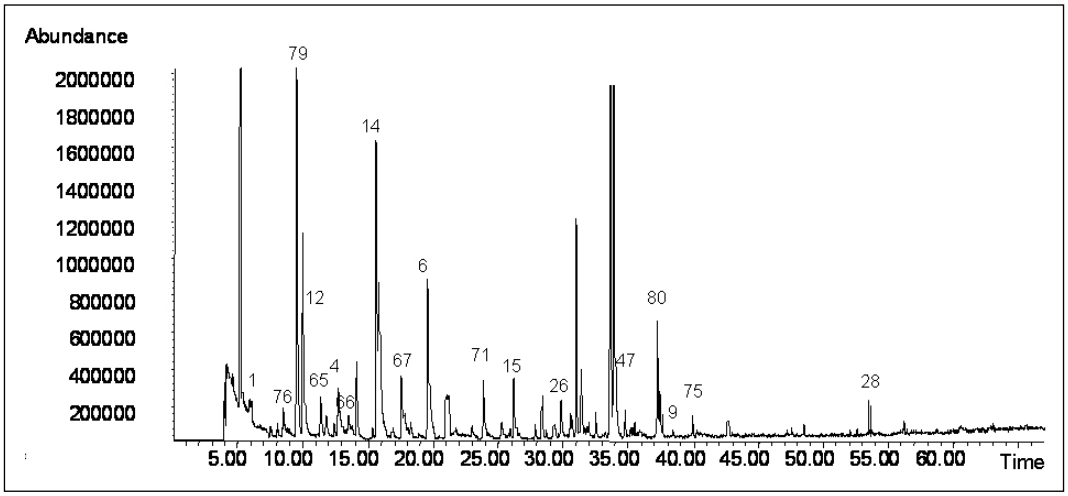


Fig. 1 - Chromatogram from headspace SPME-GC of an industrial cream (ICBP3). Peak numbers are listed in Table 1.

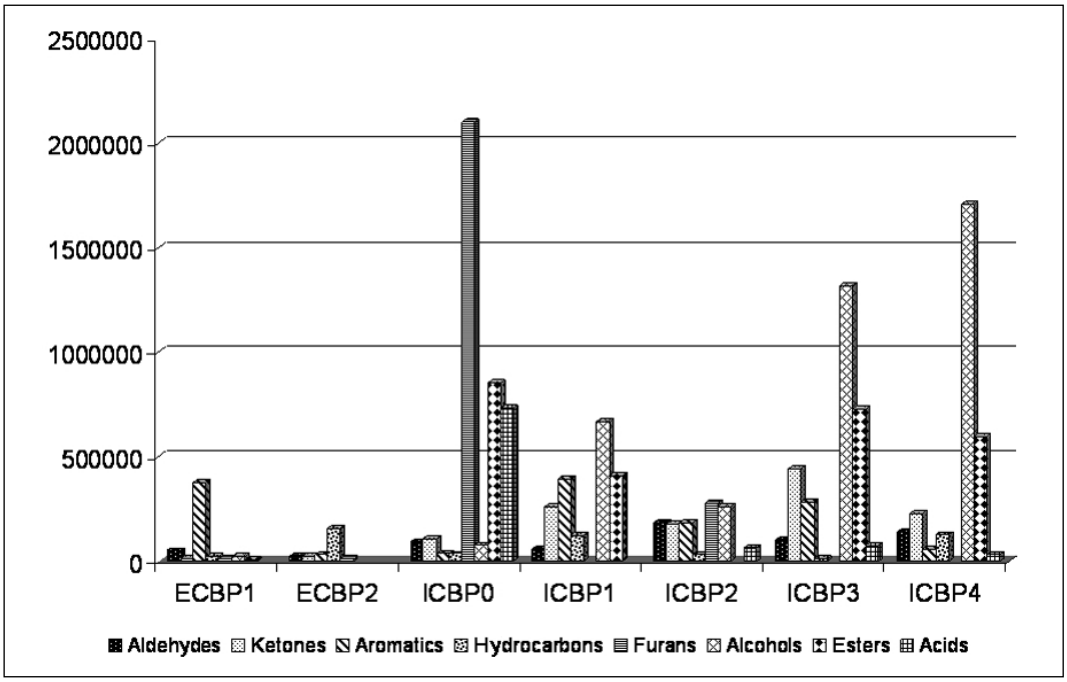


Fig. 2 - Distribution of compound classes in home-made egg creams (ECBP 1 and ECBP 2) and industrial creams (ICBP 0-4).

A straight-chain saturated alkylbenzene series was identified in ECBP 1 but not in ECBP 2 (Table 1, Fig. 2). UMANO *et al.* (1990) also reported this type of compound in heated egg. Alkylbenzenes were reported to be the product of thermal sugar degradation (HEYNS *et al.,* 1966), thus, their formation in ECBP 1 is the result of heating the sugar at higher temperatures (>110°C), whereas, in ECBP 2 sugar and milk and flour were heated at lower temperatures (<100°C) and therefore no alkylbenzenes were formed.

Toluene, described as one of the major constituents of egg volatiles (UMANO *et al.,* 1990), was detected in ECBP 1. Benzaldeyde and 1,4-xylene were detected in ECBP 1 and ECBP 2, the first one was also identified in sponge cake containing eggs and cooked egg, and the latter was reported in egg yolk heated at 50°C (POZO-BAYÓN *et al.,* 2007; STEIN and NARANG, 1990; UMANO *et al.,* 1990). 4-Hydroxy-3-methoxy-benzaldehyde results from the addition of vanillin.

Alkylfurans are well-known sugar degradation products that are also found in cooked egg and in cakes containing eggs. 5-Methylfuran-2-carbaldehyde was detected in ECBP 1 in agreement with UMANO *et al.* (1990) who detected this volatile in cooked eggs. Furan-2-carbaldeyde was detected in ECBP 2, and was also detected in sponge cake containing eggs. Several alcohols were identified in egg yolk, suggesting that alcohol formation is also associated with lipid oxidation. ECBP 1 contained 2-methylpropan-1-ol, 3-methylbutan-1-ol and 2-ethylhexan-1-ol, but no alcohol was detected in ECBP 2 (Table 1).

The Maillard reactions occurring during the cooking process are the main chemical events responsible for the generation of compounds with low odour thresholds. Different factors such as the

selection of raw materials or the modification of the making process can modify the aroma (POZO-BAYÓN *et al.*, 2007). Thus, it is understandable that ECBP 1 and ECBP 2 had different volatile profiles, since different ingredients and cooking conditions were used. ECBP 2 is made with milk (ECBP 1 with water), and this greatly affected the aroma composition for two different reasons: in the ECBP 2 the Maillard reaction could take place due to the presence of milk proteins, in ECBP 1 caramellization was the most favoured reaction, while the Maillard reaction was limited because the egg was added at 60°C.

In general, peak area values for volatile compounds in home-made egg creams (ECBP 1 and ECBP 2) were lower than those for the industrial creams (ICBP 0 to ICBP 4) (Fig. 2). The most abundant classes of compounds in ICBP 0 were furans, esters and acids. ICBP 0 showed higher peak area values than ECBPs for all classes of compounds, with the exception of hydrocarbons and aromatics (Table 1).

Concerning industrial creams collected from bakery products purchased from the market, ICBP 1 to ICBP 4 presented different qualitative and quantitative volatile profiles as can be observed in Table 1 and Fig 2. The most abundant classes of compounds in ICBP 1, ICBP 3 and ICBP 4 were alcohols and esters. Four furans were detected on ICBP 2 while no furans were detected in ICBP 1, ICBP 3 and ICBP 4. Aldehydes, hydrocarbons, alcohols and straight-chain saturated alkylbenzene usually described in egg yolk were also identified in ICBPs.

Since there were no labels describing the composition of ICBP 1-4, the major components were evaluated in the laboratory. Analysis of the major components of the four industrial cream brands revealed significant differences in the protein, fat, carbohydrate and ash contents (Table 2). These results indicate that different industrial creams are used as fillers in bakery products and therefore the volatile composition is also different.

Discriminate analysis was applied to data from the volatile profiles of ECBP 1, ECBP 2, ICBP 0, ICBP 1, ICBP 2, ICBP 3 and ICBP 4 obtained by SPME-GC/MS. Only 49 volatiles that presented a significantly different area were selected (indicated in Table 1 with an *). The discriminate analysis indicated that two functions contributed significantly to explaining the variability among the creams. The major contributors to function 1 were 2-methylpentane, hexane, octanal, 4-phenyldecane, 4-phenylundecane and 2-methylpropan-1-ol (with positive loadings), while 3-methylbutanal, heptanal, ciclopentane-1,2-dione, 3-phenyldecane and hexan-1-ol were positively correlated with function 2. Plots of the samples (Fig. 3) show two main discriminate functions that explain 94.3% of

Table 2 - Major volatile components in commercially available industrial creams.

Industrial cream g/100 g	Proteins	Fat	Carbohydrates		Ash
			Sacharose	Lactose	
ICBP1	3.2±0.3[a]	1.0±0.1[a]	38.20±1.02[a]	1.61±0.22[a]	0.7
ICBP2	3.9±0.4[a]	0.1±0.0[b]	30.86±1.53[b]	1.39±0.34[a]	0.69
ICBP3	<0.1[b]	0.2±0.1[b]	37.23±0.61[a]	n.d[b]	0.94
ICBP4	5.8±0.2[c]	4.2±0.2[c]	37.25±0.42[a]	1.76±0.31[a]	0.44

[a-c] Means in columns without common letters are significantly different (p<0.05; n=3).

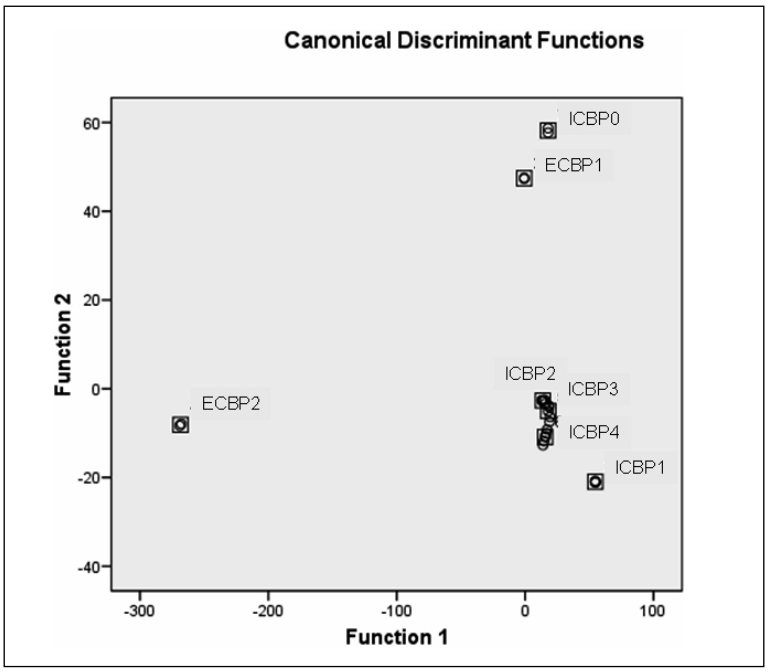


Fig. 3 - Canonical discriminate functions showing different creams used as fillers of bakery products, egg creams (ECBP 1, ECBP 2) and industrial creams (ICBP 0-4) classified by the two functions of the proposed model.

the total variance. The volatile profile of ECBP 2 could be distinguished from all the other creams analyzed. ECBP 1 and ICBP 0 had higher amounts of 3-methylbutanal, heptanal, cyclopentane-1,2-dione, 3-phenyldecane and hexan-1-ol, whereas ICBP 1-4 had higher amounts of 2-methylpentane, hexane, octanal, 4-phenyldecane, 4-phenylundecane and 2-methylpropan-1-ol. The function allowed all the samples to be classified correctly according to the type and origin of the cream.

Sensory characteristics of creams used as fillers of bakery products

The analysis of variance performed on the sensory attribute scores indicated significant differences in some of the attributes considered for ECBP 1, ECBP 2 and the ICBPs. In general, the data within each attribute were symmetric and mesocurtic. No significant differences were observed for butter aroma or strange aroma; however, significant differences were noted for all other attributes (quality of odour, sweetness, quality of aroma, egg aroma, milk aroma, flour aroma, vanillin aroma, after-taste, overall quality). The mean results obtained from 14 panellists for the 11 sensory attributes assessed in the ECBPs and ICBPs are presented in Fig. 4. Good panel performance was verified to establish the egg and industrial cream flavour descriptive profiles. ECBP 1 could be distinguished from the others due to higher values with respect to sweetness, quality of odour, quality of aroma, egg aroma and after taste. The flavour profiles of ECBP 1 and ECBP 2 were significantly different. While the sensory characteristics observed for industrial creams were

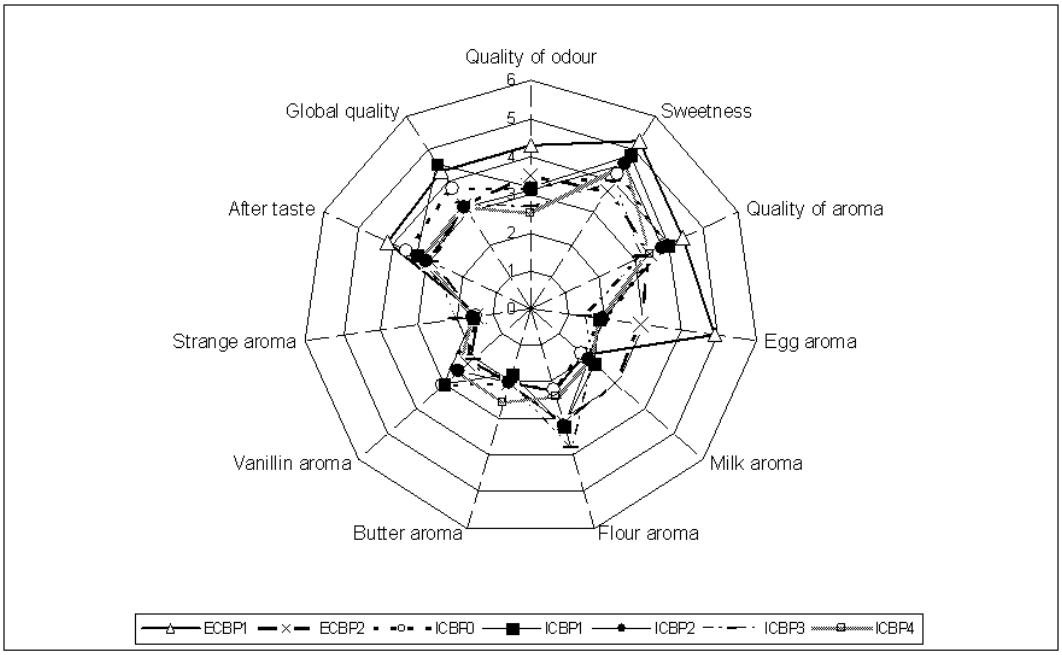


Fig. 4 - Mean scores of 11 attributes in home-made egg creams and industrial creams assessed by 14 panellists.

similar, the industrial creams ICBP 0 and ICBP 1 had flavour descriptive profiles that were more similar to that of ECBP 1, except for the egg aroma and sweetness attributes.

The Pearson correlation was applied to the sensory attributes and volatile compounds. In general, no correlations were found between the results from the descriptive flavour profile and results from the volatile compounds. However, analysis of the headspace with respect to the volatile compounds gives information about the volatiles that are present in the air above the food before eating. When the creams are eaten, many changes take place (such as hydration/dilution with saliva, increase in surface area, etc.). These changes affect the release of volatiles from the food and therefore the profile of volatiles that are sensed in the nose. The release of aromas from foods is influenced by several factors such as viscosity, interaction between aroma compounds and the presence of fat, starch and sugar (NOBLE 1996; TIETZ *et al.*, 2008). For example, when a food is eaten interactions occur between sweetness and aroma perceptions. LETHUAUT *et al.* (2005) showed that variations in aroma levels in sweetness assessment is a major factor to consider when assessing aroma. Thus, the high sweetness of ECBP 1 can interfere with aroma intensity and justify the high scores in quality of odour, quality of aroma, egg aroma and after taste for this cream.

CONCLUSIONS

HS-SPME-GC-MS is a simple, fast and sensitive method for a routine analysis of volatile compounds in cream. Headspace SPME using a 75 μm Carboxen-PDMS fibre provided effective sample enrichment and allowed a wide variety of compounds to be extracted including al-

dehydes, ketones, aromatic compounds, hydrocarbons, furans, alcohols, esters and acids.

ECBP 1 and ECBP 2 had different volatile profiles because different ingredients and different cooking conditions were used. ECBP 2 is made with milk (ECBP 1 with water) and this greatly affects aroma composition for two different reasons: in ECBP 2 the Maillard reactions could take place due to the presence of milk proteins; in ECBP 1 caramellization is the most favoured reaction, while the Maillard reaction is a limited reaction because the egg was added at 60°C.

The volatile profiles of industrial creams differed significantly from those of home-made creams. Many volatiles detected in industrial creams, including aldehydes, hydrocarbons, alcohols and straight-chain saturated alkylbenzene are also usually detected in egg yolk.

A Pearson correlation was applied to flavour attributes and volatile compounds. However, no highly significant correlations were found, because several effects, such as the presence of fat, starch and sweetness could have influenced the aroma release.

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PAPER

LACTIC ACID BACTERIA AND YEASTS FROM WHEAT SOURDOUGHS OF THE MARCHE REGION

BATTERI LATTICI E LIEVITI DA MADRI ACIDE DI FARINA DI GRANO TENERO DELLA REGIONE MARCHE

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ABSTRACT

The need for a greater diversification of baked products has given rise to the on-going search for yeast and lactic acid bacteria (LAB) strains with optimal baking potential. Thirty-six yeasts and 118 LAB, isolated from nine type I sourdoughs that were sampled in bakeries located in the Marche region (central Italy), were molecularly and phenotypically characterized. The polyphasic approach used revealed the biodiversity of the microbial communities in-

RIASSUNTO

La necessità di ampliare e diversificare l'offerta di prodotti lievitati da forno ha portato alla ricerca di ceppi di lieviti e batteri lattici con spiccate attitudini panificatorie. Trentasei lieviti e 118 batteri lattici, isolati da 9 madri acide di tipo I campionate in panifici situati sul territorio della regione Marche (Italia centrale), sono stati caratterizzati a livello molecolare e fenotipico. L'approccio polifasico utilizzato ha evidenziato la biodiversità presente nella comunità

- Key words: baking industry, lactic acid bacteria, starter cultures, wheat sourdough, yeasts -

vestigated and two yeasts and ten LAB cultures with the potential to be used in sourdough bread-making processes were identified.

microbica oggetto di studio ed ha permesso di identificare 2 colture di lievito e 10 di batteri lattici potenzialmente utilizzabili nei processi di panificazione.

INTRODUCTION

Yeasts and lactic acid bacteria (LAB) are common inhabitants of sourdoughs that originate from flour and bakery environments (DE VUYST and NEYSENS, 2005) as well as from the vegetable matter that can be added to the initial mixtures of flour and water (FOSCHINO *et al.*, 2004). A very complex ecological system is established, where different microbial species co-exist and interact in a dynamic equilibrium (GAROFALO *et al.*, 2008). This natural consortium is constantly renewed in a recycling mode under strict recipe and ripening conditions (HAMMES and GÄNZLE, 1998), and it is responsible for the so-called "sourdough fermentation" that leads to leavened baked goods with distinctive tangy or sour tastes (GÄNZLE *et al.*, 1998).

Type I sourdoughs are known to be colonized mainly by *Lactobacillus sanfraciscensis* and *Lact. pontis* (VOGEL *et al.*, 1999). Recently, DE VUYST and NEYSENS (2005) proposed a new classification of type I sourdoughs that encompasses: i) sourdoughs fermented by pasty pure starter cultures, defined as type Ia; ii) sourdoughs prepared through multiple-stage fermentation processes that contain spontaneously developed mixed cultures, defined as type Ib; and iii) sourdoughs fermented at high temperatures, and typically used in tropical regions, classified as type Ic.

In recent years, sourdough-based bread-making has been the object of renewed interest, with the ever-increasing consumer demand for tasty, more natural and healthier foods (PAGANI *et al.*, 2007). This consensus is explained by the numerous benefits of sourdough fermentation; namely, improved dough properties, crumb structure and bread texture; increased bread volume and flavour; and slower staling and prolonged mould-free shelf-life (ARENDT *et al.*, 2007). An examination of the current scientific literature shows that most of these benefits are greatly influenced by the particular yeast and lactic acid bacteria (LAB) strains that carry out the fermentation.

Commercial starters, made up of selected strains, as either single or mixed cultures, entered the market a few decades ago. The need for a greater diversification of baked products has given rise to the on-going search for strains with peculiar traits and baking potential (VOGEL *et al.*, 2002; DI CAGNO *et al.*, 2008). Although useful for maintaining the advantages of the biological fermentation process, while providing baked goods with a stable quality, the use of selected strains in sourdough biotechnology is still limited due to a generally low persistence of starters during propagation. Accordingly, the evaluation and further selection of candidate starter strains is a multi-step process that focuses on ecological, functional and technological aspects. Key criteria for this selection are based on general aspects, including origin and identity, technical aspects (growth properties *in vitro* and during processing, survival and viabil-

ity during transport, storage, etc.) and eventual functional aspects (i.e. beneficial features).

On the basis of these premises, an in-depth investigation was carried out on the microbial ecology of nine type-I wheat sourdoughs that were sampled from one semi-industrial and eight artisan bakeries of the Marche region (central Italy) using a culture-dependent approach. The main technological traits of a selected pool of yeasts and LAB were further investigated, as a basis for a preliminary selection of promising strains that could be exploited by the local baking industry for sourdough fermentation processes.

MATERIALS AND METHODS

Micro-organisms

Nine yeast (Table 1) and seven LAB (Table 2) reference strains were purchased from: (i) the Deutsche Sammlung von Mikrorganismen und Zellkulturen (DSMZ, Braunschweig, Germany); (ii) the American Type Culture Collection (ATCC, Manassas, VA, USA); (iii) the Industrial Yeasts Collection of the Department of Applied Biology, University of Perugia (DBVPG, Perugia, Italy); (iv) the National Collection of Yeast Cultures (NCYC, Norwich, UK); and (v) the Centraalbureau voor Schimmelcultures Fungal and Yeast Collection (CBS, Utrecht, The Netherlands). All of the strains were revitalized as indicated by the culture suppliers.

Sourdough sampling and pH measurements

Nine mature type I sourdoughs that had been propagated daily by back-slopping at room temperature with wheat flour were sampled in one semi-industrial (referred to as C) and eight artisan bakeries (referred to as A, B and D to I) located in the Marche region (central Italy) (Table 3). The length of the sourdough fermentation time and temperature varied considerably among the nine bakeries, according to the particular cycle of production. During ripening, the sourdoughs were bound tightly with canvas (bakeries A and C) or left to ripen in plastic containers (bakeries B, and D to I).

In the semi-industrial bakery C, compressed bakers' yeast had never been used before. In bakeries A, G and H, it was routinely used in the production lines that are different from those considered in the present study, while in bakeries B, D, E, F and I, the sourdoughs sampled had been prepared with trace amounts of this leavening agent. The compressed bakers' yeast used by bakeries A, B, and D to I were sampled and used for the isolation of the industrial yeast strains, referred to as the controls in both the molecular typing and the technological characterization. All of the samples were kept under refrigerated conditions and analysed within 24 h. Only the sourdough samples collected from bakeries A, C, G and H were used further for the isolation of sourdough yeasts because their preparations did not include the addition of compressed bakers' yeast.

The pH measurements of the sourdough samples were carried out in duplicate with a model 300 pH meter (Hanna Instruments, Padova, Italy) equipped with an HI2031 solid electrode (Hanna Instruments).

Microbial counting and isolation of yeasts and LAB

Approximately 10 g of each sourdough sample were diluted in 90 mL of a sterile peptone water solution (1 gL^{-1} peptone, 8.5 gL^{-1} NaCl) and homogenised for 2 min at 260 rpm using a Stomacher apparatus (400 Circulator, PBI International, Milan, Italy). The homogenates were serially diluted and aliquots (100

Table 1 - Yeast reference strains used for the construction of the RFLP pattern database.

Species	Reference strain	Amplicon size (bp)	Restriction fragments (bp)		
			CfoI	*HaeIII*	*HinfI*
Issatchenkia orientalis	DSM3433	510	210-180-80	380-90	200-150-130
Pichia angusta	NCYC495	750	300-240-120-90	540-210	300-400
Candida milleri	DBVPG6753	790	330-330-120	790	350-200-120-70
Pichia anomala	DBVPG6613, DBVPG4357, DBVPG6781	650	550	650	310-310
Saccharomyces cerevisiae	CBS4054, CBS1171	880	370-350-140	330-230-180-140	365-365-140
Starmerella bombicola	DBVPG3827	475	220-190	325-80	235-235

Table 2 - Lactic acid bacteria reference strains and GenBank 16S rRNA gene sequences used for the construction of the ARDRA pattern database.

Species	GenBank Acc. no.	Strain	Amplicon size (bp)	Restriction fragments (bp)		
				AluI	*FokI*	*HaeIII*
Lactobacillus alimentarius	M58804	DSM20249	360	95-251	122-243	314
Lact. paralimentarius	AJ417500; AJ422034	N.A.	360	69-96-182	123-222	314
Lact. panis	X94230	DSM6035	360	183-237	191-229	78-120-191
Lact. pontis	X76329; AJ422033	DSM8475	360	114-226	122-232	51-78-191
Lact. sanfranciscensis	X76331; X76327	DSM20451	360	95-217	122-254	126-221
Lact. fructivorans	X76330	N.A.	360	52-114-179	121-253	104-220
Lact. lindneri	X95421	N.A.	360	67-96-179	123-254	347
Weissella confusa	AB023241	ATCC14434; DSM20196	360	57-115-188	360	308
W. viridescens	AB023236	DSM20410	360	57-115-188	360	308
W. kimchii	AF515221; AF312874	N.A.	360	75-304	358	327

N.A. not available.

Table 3 - Type I sourdough samples collected in one semi-industrial and eight artisan bakeries of the Marche region (central Italy).

Bakery	Location (District)	Ripening time (h)	Ripening temperature (°C)	pH*	Log cfu g^{-1}	
					Yeasts	LAB
A	Ancona	20	20	3.80±0.02	7.0	8.3
B	Pesaro-Urbino	22	Troom	3.15±0.01	7.4	8.6
C	Pesaro-Urbino	24	Troom	3.85±0.01	7.7	9.2
D	Ancona	4-5	20-25	3.80±0.01	7.6	8.4
E	Macerata	15-17	8-10	4.20±0.02	8.0	8.5
F	Macerata	24	Troom	4.27±0.02	7.4	8.6
G	Ascoli Piceno	20	Troom	4.45±0.01	6.9	8.6
H	Ascoli Piceno	48	25	3.80±0.02	7.1	9.0
I	Ascoli Piceno	7-8	25	3.90±0.01	8.0	9.0

* Mean value ± standard deviation.

μL) of each dilution were streaked onto the agar media listed below.

The yeasts were counted and isolated on Wallerstein Laboratory Nutrient (WLN) agar (Oxoid, Basingstoke, UK) with 250 mg L^{-1} chloramphenicol added (GOBBETTI *et al.*, 2000) and incubated at 25°C for 96 h. Up to three colonies were selected according to each morphology and colour on the WLN plates, and they were streaked to purity onto the same medium. For each isolate, the cell morphology was examined using a light microscope under oil-immersion (100x). The yeast isolates were stored frozen (-80°C) in a mixture of glycerol and YPD (Oxoid) (1:1).

The LAB were counted on: (i) De Man, Rogosa and Sharp (MRS) agar (DE MAN *et al.*, 1960), modified by the addition of 1% maltose and 5% fresh yeast extract (mMRS) (GOBBETTI *et al.*, 1996) (ii) sourdough medium (SDB) (KLINE and SUGIHARA, 1971); and (iii) GM17 agar (HORN *et al.*, 1999). To inhibit the yeast growth 250 mg L^{-1} cycloheximide were added to these three media. The incubations were carried out at 30°C under anaerobic conditions (Anaerogen GasPak System, Oxoid, Basingstoke, UK) for 48-72 h. After counting, LAB colonies were randomly selected and picked from the last dilution plates; the bacterial isolates were tested for Gram and catalase reactions, and stored frozen (-80°C) in a mixture of glycerol and mMRS (1:1).

Molecular identification of yeasts and LAB

The yeast isolates were initially identified by restriction fragment length polymorphism (RFLP) analysis. To widen the existing 5.8S-ITS restriction pattern database that is available for the identification of food-borne yeasts, *in-silico* restriction of nucleotide sequences retrieved form the GenBank DNA database was performed with three endonucleases *Cfo*I, *Hae*III and *Hinf*I and the TACG software, which is available at http://bioweb.pasteur.fr/seqanal/interfaces/tacg.html.

In the experimental RFLP analyses, the nine reference yeast strains listed in Table 1 were used as controls. The DNA was extracted from YPD broth cultures as described by MAKIMURA *et al.* (1999). Aliquots (1 μL) of the template DNA were amplified according to ESTEVE-ZARZOSO *et al.* (1999). Twenty μL of the PCR products were digested separately in 30 μL reaction volumes with 2 U of *Cfo*I, *Hae*I-

II and *Hinf*I (Roche Diagnostics, Germany). The digests were analysed by electrophoresis in 2.5% (w/v) agarose gels at 3.5 V cm^{-1} constant voltage for 3 h. A 50-bp DNA size marker (Amersham Biosciences, Amersham, UK) was used for size standards.

The LAB isolates were initially identified by amplified rDNA restriction analysis (ARDRA). An *in-silico* restriction analysis similar to that performed with the 5.8S-ITS nucleotide sequences was carried out on the 16S rRNA gene sequences retrieved from the GenBank DNA database and ascribed to LAB species commonly found in flour and sourdough.

In the experimental ARDRA assays, seven reference strains (listed in Table 2) were used as controls. The bacterial DNA was extracted from the SDB broth cultures as described by DE LOS REYES-GAVILÀN *et al.* (1992). Quantity and purity of the DNA were assessed by optical reading at 260 and 280 nm, respectively (SAMBROOK *et al.*, 1989). An approximately 360-bp portion of the 16S rRNA gene was amplified using universal primers for eubacteria, and separately digested with *Fok*I, *Hae*III and *Alu*I (Roche Diagnostics, Mannheim, Germany), as previously described (AQUILANTI *et al.*, 2007).

The gels were stained with ethidium bromide and photographed under UV light. The electronic images of the gels were visualized with the ImageMaster VDS apparatus (Amersham Pharmacia), captured with LISCAP software v.1.0 (Amersham Pharmacia), and stored as TIFF files. The digitalized images were normalized with the 50-bp DNA size marker (Amersham Pharmacia), and analyzed with GelCompar, v. 4.0 (Applied Maths, Kortrijk, Belgium).

The isolates were grouped by comparing their restriction patterns to those included in published RFLP (GUILLAMÓN *et al.*, 1998; DLAUCHY *et al.*, 1999; ESTEVE-ZARZOSO *et al.*, 1999; ARIAS *et al.*, 2002; GULLO *et al.*, 2003; PULVIRENTI *et al.*, 2004) and ARDRA (AQUILANTI *et al.*, 2007) databases, as well as those of the databases built specifically for the present study. The diagnosis of species was verified by sequencing of the amplicons (600-800 bp for yeasts, 360 bp for LABs) from one or more isolates selected within each RFLP and ARDRA group. The PCR products were purified using micro-columns (GFX purification kit, Amersham Biosciences), according to the manufacturer's instructions, and sent to MWG Biotech (Milan, Italy) for sequencing. The closest relatives of the sequences obtained were determined through searches within the GenBank DNA database using the BLAST algorithm (ALTSCHUL *et al.*, 1990).

The 54 bacterial isolates assigned to *Lact. plantarum sensu latu* underwent *recA* multiplex PCR assay, as described by TORRIANI *et al.* (2001), for a finer discrimination at the species level. The 11 bacterial isolates for which the genotype-based identification led to an ambiguous diagnosis underwent the following phenotype-based tests: fermentation of sucrose, melibiose, L-arabinose, trehalose, raffinose and lactose (evaluated using a miniaturized assay in microtiter plates) and hydrolysis of arginine, in modified MRS broth medium (AQUILANTI *et al.*, 2007).

Molecular typing of yeasts

Three μL of the total genomic DNA extracted from the sourdough and compressed bakers' yeast isolates were used in the PCR reactions, as described by MAKIMURA *et al.* (1999). Inter-delta sequences were amplified as described by CIANI *et al.* (2004).

Technological characterization of the isolates

The LAB were sub-cultured in SDB medium and incubated at 30°C for 24

h, while the yeasts were sub-cultured in YPD broth (Oxoid) and incubated at 25°C for 24 h.

For the assessment of the LAB acidifying activities and the CO_2 production from yeasts and LAB, an inoculum standardisation was required. Accordingly, stationary phase cells were harvested by centrifugation at 4,200 x *g*, washed twice, and resuspended in sterile distilled water. The cell suspensions were diluted to an optical density at 620 nm (OD_{620}) of 1.25 which, according to CORSETTI *et al.* (1998), corresponded to yeast and LAB counts of 10^7 and 10^9 cfu mL^{-1}, respectively. The standardised suspensions were used as a 4% (v/v) inoculum in the first two assays, following.

LAB acidifying activity

The acidifying activity of the 118 LAB isolates were determined in SDB broth (initial pH, 5.6), incubated at 30°C under aerobic conditions. After 6 and 24 h of fermentation, 1 mL samples were aseptically withdrawn for pH assessment using a model 9224 pH meter (Hanna Instruments, Padova, Italy) equipped with a microtube electrode (Hamilton, Reno Nevada, USA). For the 34 pre-selected LAB isolates ascribed to species of technological interest for the baking industry, the amount of lactic acid and acetic acid produced after a 24 h incubation was determined spectrophotometrically, using two commercially available enzymatic kits (Kit Nos. 11112821035 and 0148261, respectively; Boehringer Mannheim/ R-Biopharm, Darmstadt, Germany).

Yeast and LAB CO_2 production

The amount of CO_2 produced by 36 sourdough yeasts and 34 pre-selected heterofermentative LAB isolates was evaluated by inoculating the cell suspensions into 100 mL flasks containing 70 mL SDB broth. After the inoculation, the flasks were aseptically sealed with Müller valves, which allowed the CO_2 to escape the system (CIANI and ROSINI, 1987). The flasks were weighed immediately after inoculation and after 24 h incubation at 25°C for yeasts, and at 30°C for LAB, using an analytical balance (Analytical Plus, Ohaus, New Jersey). The CO_2 produced was expressed as the weight loss of the fermented broths after this 24 h fermentation.

Yeast and LAB amylase activity

The amylase activities of 36 sourdough yeasts and 34 pre-selected LAB isolates were qualitatively determined using the method described by SEELEY *et al.* (1995). Starch hydrolysis was revealed by the appearance of clear halos surrounding the colonies exposed to a 0.25% iodine solution (DUNGA *et al.*, 2006). For the LAB isolates that could hydrolyse starch, the amylase activities were quantitatively determined as follows: twenty µL of the standardised cell suspensions were inoculated into 180 µL of 0.1 mol L^{-1} phosphate buffer (pH 7.0) containing 10 g L^{-1} soluble starch (Sigma-Aldrich, Milan, Italy). After incubation at 30°C for 3 h, the residual concentrations of starch were determined using an enzymatic kit (Kit No. 207748 Boehringer Mannheim/R-Biopharm), according to the manufacturer instructions.

Statistical analysis

Three replicates of each technological assay were performed. Arithmetic means and standard deviations were calculated. One-way analysis of variance (ANOVA) and the Tukey Kramer honestly significant difference (HSD) based on three replicates were carried out using the JMP software package (version 3.15, S.a.s. Institute Inc. Carry, NC, USA), with the following variables: VAR1, species, VAR2, isolates within the same species; VAR3, acidifying activity at 6 h fermentation; VAR4, acidifying activity at 24-h fermentation; VAR5, lactic-acid production;

VAR6, acetic-acid production; VAR7, CO_2 production; and VAR8, amylase activity.

RESULTS

Sourdough pH measurements, microbial counting and isolation

Nine mature type I sourdough samples were collected from nine bakeries in the Marche region (central Italy). For the sourdough acidification, the pH values ranged from 3.15 to 4.45 (Table 3).

The viable microbial counts showed that the sourdough samples contained yeasts and LAB in ratios that varied from about 1:100 (samples collected from bakeries C, G and H) to 1:10 (samples collected from bakeries A, B, D to F) (Table 3). The yeast cell numbers ranged from log 6.9 to log 8.0 cfu g^{-1}, while those of the LAB varied from log 8.3 to log 9.2 cfu g^{-1}.

The yeasts were isolated only from the sourdough samples collected from bakeries A, C, G and H, the refreshment of which did not include the direct addition of compressed bakers' yeast, while the LAB were isolated from all of the sourdough samples. The isolation campaign yielded 36 sourdough yeast and 118 bacterial isolates. Eleven yeast cultures were isolated the compressed bakers' yeast, that is commonly used in bakeries A, B, D to I. Remarkable morphological differences in colony colour, elevation, surface and edge were seen in the yeast colonies grown on WLN agar (data not shown). All of the bacterial isolates were Gram positive and catalase negative.

Yeast and LAB molecular identification

For the RFLP analysis, the amplification of the 5.8S-ITS region from the reference strains and the isolates yielded PCR products that were characterized by a high degree of length variation. The sizes of the PCR amplicons and of the *Cfo*I, *Hae*III and *Hinf*I digests are shown in Table 2. For all of the yeast reference strains, the experimental patterns were comparable to those obtained through the *in-silico* simulation. For the 36 yeast isolates, 33 were characterized by restriction patterns identical to those produced by the two *S. cerevisiae* reference strains, CBS1171T and CBS4054. The remaining three isolates were ascribed to a different group, since they showed restriction profiles that were not comparable to those collected in the present and other published databases (FOSCHINO *et al.*, 2004; ARIAS *et al.*, 2002; GULLO *et al.*, 2003; PULVIRENTI *et al.*, 2004). Consequently, the 5.8S-ITS amplicons had to be sequenced in order to have an unequivocal identification of these isolates. The alignment of these sequences with those published for the species *Candida humilis* (Acc. no. AY188851) resulted in identities that exceeded 98%, thus confirming the identification of these yeasts. For the ARDRA analysis, PCR products of the expected sizes were obtained from both the LAB reference strains and the isolates. The sizes of the *Fok*I, *Hae*III and *Alu*I digests are shown in Table 2. The restriction profiles generated by the LAB reference strains were consistent with those obtained through the *in-silico* simulation (Table 2). A large proportion of the most abundant LAB species in the sourdoughs was readily differentiated from each other. On the other hand, two species, *Weissella confusa* and *W. viridescens*, could not be differentiated after digestion with *Fok*I, *Hae*III and *Alu*I.

Restriction profile comparisons, partial sequencing of the 16S rRNA gene, and *recA* multiplex PCR assays allowed 107 isolates to be unambiguously identified at the species level. For the remaining 11 isolates, further phenotype-based analyses were performed, thus allowing their definitive assignment to species.

The microbial map of the nine sourdoughs is shown in Fig. 1. A high degree of biodiversity was seen across the ecosystems investigated. *Lact. plantarum* and

Bacteria	Bakery A	B	C	D	E	F	G	H	I	No. isolates
Lact. plantarum	■(6)	■(3)	■(6)	■(6)	■(4)	■(3)	■(6)	■(9)	■(11)	54
Lact. curvatus	■(2)	■(2)		■(3)						7
Lact. sakei		■(1)								1
Lact. rhamnosus			■(1)							1
Lact. helveticus								■(2)		2
Lact. gallinarum									■(1)	1
Lact. sanfranciscensis	■(3)	■(4)					■(1)			8
Lact. paralimentarius			■(7)			■(3)		■(1)	■(1)	12
Lact. brevis						■(5)				5
Lact. casei ssp. carnosus	■(1)									1
Lact. paracasei ssp. paracasei							■(1)	■(1)		2
Lact. fermentum					■(1)	■(2)				3
Lact. casei sensu latu	■(1)									1
S. salivarius		■(1)		■(1)						2
Leuc. citreum	■(1)	■(1)								2
Leuc. pseudomesenteroides							■(9)	■(1)		10
W. confusa				■(1)	■(2)	■(1)				4
W. cibaria				■(1)						1
E. faecalis									■(1)	1
									Total	**118**
Yeasts										
S. cerevisiae	■(6)		■(10)				■(7)	■(10)		33
C. humilis	■(3)									3
									Total	**36**

Fig. 1 - Microbial map of the nine sourdoughs from the Marche region (central Italy) under study. For each sourdough sample, the number of isolates ascribed to yeast and LAB species are reported in brackets.

Saccharomyces cerevisiae were present in all of the sourdough samples, followed by *Lact. paralimentarius* (4 samples), *Lact. curvatus* (3 samples), *Lact. sanfranciscensis* (3 samples) and *W. confusa* (3 samples).

Yeast molecular typing

The amplification of the inter-delta sequences from the 36 sourdough and 11 bakers' yeast isolates allowed the identification of seven fingerprints, referred to as α to η (Fig. 2). The first four amplification patterns (α, β, γ, δ) were obtained from the sourdough isolates, while the latter three (ε, ζ, η) were obtained from the industrial strains. Comparative evaluation of these fingerprints showed the genetic diversity of the sourdough isolates (Table 4). For nine isolates, the amplification of the inter-delta sequences did not allow the visualization of any PCR products. This is not unusual for *S. cerevisiae* (EGLI *et al.*, 1998; CIANI *et al.*, 2004; MARINANGELI *et al.*, 2004). Regarding the sources of the isolates, the sourdough sample collected in bakery C was characterised by the highest strain

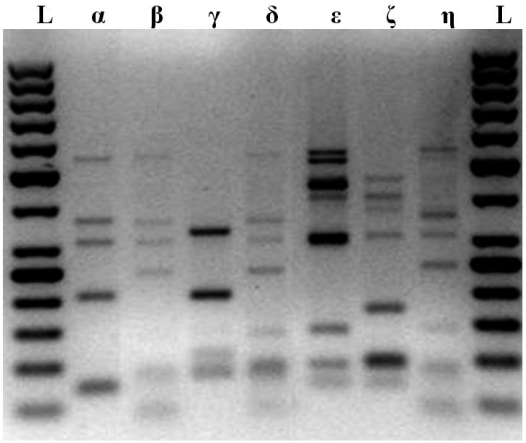


Fig. 2 - Inter-delta amplification profiles generated by PCR from the 36 sourdough and 11 industrial *Saccharomyces cerevisiae* isolates.
L- Molecular weight marker (100 bp DNA Ladder, M-Medical, Milan, Italy).

Table 4 - Technological characterization and molecular typing of the 36 sourdough yeast isolates.

Bakery	Isolate	Species	Amyl.	CO_2 (g L^{-1})			Inter-delta fingerprint
A	PL11	*C. humilis*	-	0.01	o	(0.00)	N.D.
	PL12		+	0.20	o	(0.00)	N.D.
	PL13		-	0.50	n	(0.01)	N.D.
	PL15	*S. cerevisiae*	-	1.35	l	(0.01)	*
	PL16		++	3.17	ef	(0.01)	*
	PL17		++	3.18	ef	(0.02)	*
	PL18		+	2.30	hi	(0.01)	*
	PL19		++	3.13	f	(0.02)	*
	PL20		++	2.54	g	(0.03)	*
C	PL31		+	3.63	de	(0.01)	*
	PL32		-	3.72	de	(0.02)	α
	PL33		+++	4.50	b	(0.04)	α
	PL34		+/-	2.67	fg	(0.02)	α
	PL35		++	N.D.	N.D.	N.D.	α
	PL36		+	3.63	de	(0.03)	*
	PL37		-	4.08	c	(0.04)	*
	PL38		++	N.D.	N.D.	N.D.	α
	PL39		-	N.D.	N.D.	N.D.	α
	PL40		+	3.29	e	(0.02)	β
G	PL76		+/-	N.D.	N.D.	N.D.	γ
	PL77		+/-	0.65	n	(0.00)	γ
	PL78		+/-	N.D.	N.D.	N.D.	γ
	PL79		+++	2.69	fg	(0.01)	δ
	PL80		+++	2.77	fg	(0.01)	δ
	PL82		++	3.25	e	(0.02)	δ
	PL83		+++	2.38	gh	(0.02)	δ
H	PL94		+++	4.13	b	(0.05)	α
	PL95		+++	N.D.	N.D.	N.D.	α
	PL96		++	2.73	gh	(0.01)	α
	PL97		+++	4.70	a	(0.04)	α
	PL98		+++	N.D.	N.D.	N.D.	α
	PL99		+/-	2.49	g	(0.01)	α
	PL100		+/-	2.16	hi	(0.02)	α
	PL101		+/-	3.76	de	(0.02)	α
	PL102		+/-	N.D.	N.D.	N.D.	α
	PL103		+++	N.D.	N.D.	N.D.	α

For CO_2 production, the mean values are expressed in g L^{-1} of gas released after 24 h. Standard deviations (±) are reported in brackets, while different letters denote significant differences ($P<0.05$). * no amplification; N.D. Not Determined; - no hydrolysis; + low hydrolysis; ++ medium hydrolysis; +++ high hydrolysis.

diversity (two fingerprints and three isolates that did not generate any amplification products), while apparently genetically homogeneous populations of *S. cerevisiae* were seen for the sourdough samples from bakery A and H.

Technological characterization

LAB acidifying activity

The LAB acidification kinetics were assessed after 6 and 24 h of fermentation (data not shown). For each sour-

dough sample, the isolate showing the highest acidifying activity among those ascribed to the same species was chosen and taken through further technological assays. This preliminary screening allowed 34 isolates to be pre-selected (Table 5). Although the ability to rapidly decrease the pH of the SDB medium significantly varied among the isolates, the majority of them showed pH values lower than 5.00 after only 6 h of fermentation. Among these, the isolates *Lact. plantarum* PB11 and *Lact. sakei* PB14 stood out as having significantly greater acidifying velocities.

Production of L/D-lactic acid and acetic acid

The amounts of L/D-lactic acid and acetic acid produced by the 34 pre-selected LAB isolates are shown in Table 5. As it has emerged from the statistical evaluation of these two chemometric parameters, the isolate *Lact. plantarum* PB11 differed significantly from the others; it had the highest lactic acid production (6.28 gL^{-1}). On the other hand, the isolates *Lact. plantarum* PB210 and PB46, as well as *Lact. sanfranciscensis* PB211 and *Leuc. citreum* PB220, had the highest acetic acid production values.

CO_2 production

The CO_2 production from the 36 sourdough yeasts and 34 pre-selected heterofermentative LAB was evaluated (Tables 4 and 5). For the majority of the LAB, the amount of CO_2 produced was between 0.20 and 1.90 g L^{-1}, although it ranged from 3.5 g L^{-1} (detected for *Lact. plantarum* PB11) to 0.2 g L^{-1} (detected for *Lact. paralimentarius* PB94). For the yeasts, the isolates belonging to *Candida humilis* showed CO_2 production between 0.01 and 0.50 g L^{-1}, while for those ascribed to *S. cerevisiae*, this ranged from 0.65 to 4.70 g L^{-1}. Within this latter group, values comparable to those shown by the 11 *S. cerevisiae* strains isolated from the bakers' yeast (2.6-5.0 gL^{-1}) were seen in 17 cultures. Two isolates (*S. cerevisiae* PL97 and PL33) showed the best performance, releasing 4.70 and 4.50 g L^{-1} CO_2, respectively. Both these genera had high variabilities as noted by ANOVA (Table 5).

Starch hydrolyses

The 36 sourdough yeasts and 34 preselected LAB were first screened on MRS-starch plates for qualitative assessment of their amylolytic abilities. For the yeasts, the ability of the isolates ascribed to *S. cerevisiae* to hydrolyse starch varied from (-) to (+++), while a negative amylolytic ability was seen for all of the isolates belonging to *Candida*, except for the isolate PL12 (Table 4). For the LAB isolates that showed a positive activity (from + to +++), a quantitative determination of the starch hydrolysis was conducted (Table 5). Among the LAB, only nine isolates were able to hydrolyse starch. When the amounts of hydrolysed starch, which ranged from 1.10 to 2.13 g L^{-1}, was statistically evaluated, a wide inter- and intra-specific variability was seen (Table 5). While several *Lact. plantarum* isolates were characterized by a high amylolytic capacity, the *W. confusa* PB150 isolate had the best performance.

Definition of LAB and yeast biotypes

By considering the species, the source of isolation and the results of the technological characterization (plus the molecular fingerprinting, when available), almost all of the 36 yeast and 34 preselected LAB isolates were bio-diverse, except for two clones (*S. cerevisiae* PL79 and PL80) identified among the yeasts.

DISCUSSION

The industrialization of the baking process has led to an increased demand for defined single-strain and multi-strain culture preparations for standardised sourdough fermentations (GOB-

Table 5 - Technological characterization of the 34 LAB isolates from type I sourdoughs of the Marche region (central Italy).

Bakery	Isolate	Species	Acidification						Amy. activity	Starch			Primary metabolites (gL^{-1})								
			6 h			24 h				hydrolysed (gL^{-1})			Lactate			Acetate			CO_2		
A	PB5	*Lact. casei sensu latu*	3.69	c	(0.01)	3.27	b	(0.01)	-	N.D.		N.D.	3.20	de	(0.02)	0.06	b	(0.00)	1.10	d	(0.01)
	PB1	*Lact. curvatus*	4.24	i	(0.02)	4.12	g	(0.01)	-	N.D.		N.D.	3.33	d	(0.03)	0.05	b	(0.00)	1.20	cd	(0.01)
	PB210	*Lact. plantarum*	4.04	g	(0.01)	3.48	cd	(0.01)	+	1.12	m	(0.01)	2.62	de	(0.04)	0.13	a	(0.02)	0.92	d	(0.01)
	PB211	*Lact. sanfranciscensis*	5.27	q	(0.02)	3.82	f	(0.01)	+++	1.73	f	(0.01)	2.37	f	(0.01)	0.13	a	(0.03)	0.95	d	(0.02)
	PB3	*Leuc. citreum*	3.81	ef	(0.01)	3.54	cd	(0.01)	+++	1.94	c	(0.02)	2.33	ef	(0.01)	0.10	ab	(0.01)	1.00	d	(0.01)
B	PB115	*Lact. curvatus*	4.13	h	(0.02)	3.71	e	(0.01)	-	N.D.		N.D.	5.32	ef	(0.01)	0.09	ab	(0.01)	1.90	b	(0.02)
	PB11	*Lact. plantarum*	3.43	a	(0.01)	3.14	a	(0.05)	-	N.D.		N.D.	6.28	a	(0.02)	0.10	ab	(0.02)	3.50	a	(0.01)
	PB14	*Lact. sakei*	3.52	b	(0.01)	3.25	b	(0.01)	-	N.D.		N.D.	3.33	de	(0.04)	0.08	ab	(0.00)	1.12	d	(0.01)
	PB223	*Lact. sanfranciscensis*	4.05	g	(0.03)	3.46	cd	(0.03)	-	N.D.		N.D.	2.11	ef	(0.01)	0.07	ab	(0.01)	0.86	d	(0.01)
	PB220	*Leuc. citreum*	4.52	l	(0.02)	3.68	e	(0.08)	-	N.D.		N.D.	2.02	fg	(0.02)	0.13	a	(0.01)	0.70	e	(0.01)
C	PB23	*Lact. paralimentarius*	3.93	c	(0.01)	3.54	cd	(0.01)	-	N.D.		N.D.	2.88	de	(0.04)	0.06	b	(0.00)	1.20	cd	(0.04)
	PB233	*Lact. rhamnosus*	4.26	l	(0.01)	3.65	e	(0.09)	-	N.D.		N.D.	3.00	de	(0.06)	0.01	b	(0.00)	1.45	c	(0.02)
D	PB33	*Lact. curvatus*	3.92	g	(0.01)	3.65	e	(0.01)	+++	1.47	h	(0.03)	2.10	ef	(0.01)	0.05	b	(0.00)	0.75	e	(0.02)
	PB24	*Lact. plantarum*	3.68	cd	(0.05)	3.21	ab	(0.02)	-	N.D.		N.D.	2.96	de	(0.01)	0.07	ab	(0.00)	1.10	d	(0.03)
	PB242	*Lact. plantarum*	3.84	f	(0.02)	3.50	cd	(0.03)	-	N.D.		N.D.	1.45	g	(0.01)	0.03	b	(0.00)	0.50	e	(0.02)
	PB243	*Lact. plantarum*	3.83	f	(0.03)	3.44	cd	(0.01)	+++	1.83	e	(0.05)	2.19	ef	(0.01)	0.10	ab	(0.01)	0.80	d	(0.01)
	PB32	*W. cibaria*	3.62	d	(0.03)	3.52	cd	(0.01)	-	N.D.		N.D.	2.92	ef	(0.01)	0.06	b	(0.00)	1.11	d	(0.03)
E	PB46	*Lact. plantarum*	3.75	de	(0.01)	3.28	b	(0.01)	++	1.46	h	(0.02)	2.12	ef	(0.03)	0.13	a	(0.02)	0.92	d	(0.02)
	PB150	*W. confusa*	4.86	n	(0.02)	4.29	h	(0.02)	+++	2.13	a	(0.03)	2.62	ef	(0.04)	0.10	ab	(0.02)	1.10	d	(0.01)
F	PB 55	*Lact. brevis*	3.88	f	(0.01)	3.46	cd	(0.01)	-	N.D.		N.D.	2.43	ef	(0.01)	0.12	a	(0.01)	0.90	d	(0.01)
	PB162	*Lact. fermentum*	4.23	i	(0.04)	3.45	cd	(0.02)	-	N.D.		N.D.	3.10	de	(0.01)	0.08	ab	(0.01)	1.15	cd	(0.03)
	PB264	*Lact. paralimentarius*	4.06	h	(0.03)	3.66	e	(0.02)	-	N.D.		N.D.	2.71	ef	(0.02)	0.05	b	(0.00)	1.10	d	(0.03)
	PB268	*Lact. plantarum*	4.14	h	(0.03)	3.42	c	(0.03)	+	1.10	n	(0.01)	2.02	gf	(0.01)	0.12	a	(0.01)	0.65	e	(0.01)
G	PB284	*Lact. paracasei* ssp. *paracasei*	4.04	g	(0.01)	3.56	d	(0.02)	-	N.D.		N.D.	1.24	g	(0.08)	0.03	b	(0.00)	0.47	e	(0.01)
	PB277	*Lact. plantarum*	3.84	f	(0.08)	3.44	c	(0.07)	-	n.d.		n.d.	4.20	c	(0.01)	0.10	ab	(0.02)	1.50	c	(0.00)
	PB287	*Lact. plantarum*	5.17	p	(0.01)	3.71	e	(0.01)	+++	1.98	b	(0.04)	3.32	de	(0.02)	0.05	b	(0.01)	0.45	e	(0.01)
	PB276	*Lact. sanfranciscensis*	4.13	h	(0.03)	3.50	cd	(0.02)	+++	1.72	g	(0.01)	3.52	d	(0.06)	0.05	b	(0.01)	1.35	cd	(0.01)
	PB172	*Leuc. pseudomesenteroides*	4.84	h	(0.04)	3.75	e	(0.10)	-	N.D.		N.D.	2.61	ef	(0.01)	0.08	ab	(0.01)	1.10	d	(0.02)
	PB288	*Leuc. pseudomesenteroides*	5.09	op	(0.01)	3.80	ef	(0.01)	-	N.D.		N.D.	1.78	fg	(0.01)	0.04	b	(0.01)	0.65	e	(0.01)

Continued tab. 5.

Bakery	Isolate	Species	Acidification						Amy. activity	Starch	Primary metabolites (gL^{-1})							
			6 h			24 h				hydrolysed (gL^{-1})	Lactate			Acetate			CO_2	
H	PB294	*Lact. paracasei* ssp. *paracasei*	5.05	o	(0.01)	3.43	c	(0.03)	-	N.D.	N.D.	1.31	g	(0.07)	0.06	b	(0.01)	0.46	e	(0.00)
	PB94	*Lact. paralimentarius*	4.02	g	(0.04)	3.50	cd	(0.01)	-	N.D.	N.D.	1.03	g	(0.01)	0.04	b	(0.00)	0.20	e	(0.01)
	PB193	*Lact. plantarum*	5.38	r	(0.01)	3.36	bc	(0.04)	-	N.D.	N.D.	2.89	ef	(0.01)	0.06	b	(0.01)	1.10	d	(0.01)
	PB202	*Lact. plantarum*	4.97	o	(0.01)	3.33	b	(0.02)	-	N.D.	N.D.	2.90	ef	(0.06)	0.04	b	(0.00)	1.00	d	(0.01)
	PB295	*Leuc. pseudomesenteroides*	4.02	g	(0.01)	3.48	cd	(0.02)	-	N.D.	N.D.	2.56	ef	(0.01)	0.05	b	(0.01)	1.10	d	(0.01)

For the quantitative assays, mean values are expressed as: pH measured after 6 and 24 h; gL^{-1} of hydrolyzed starch; gL^{-1} of lactate and acetate produced after 12 h; gL^{-1} of CO_2 produced after 24 h. Within each dataset, standard deviations (±) are reported in brackets, while different letters denote significant differences ($P < 0.05$).
N.D.: not detected.
-: no hydrolysis; +: low hydrolysis; ++: medium hydrolysis; +++: high hydrolysis; ++++: very high hydrolysis.

BETTI *et al.*, 1995). The strain selection for industrial applications relies greatly on the evaluation of distinctive microbial activities for improved sourdough bread quality (GOBBETTI and GÄNZLE, 2007). Each sourdough is a unique ecosystem, where a balanced population of yeasts and LAB interact in a dynamic equilibrium. Accordingly, the selection of the resident sourdough strains to be used as starters requires prior knowledge of the complexity and structure of this ecosystem. Indeed, the successful use of starters largely depends on competition with autochthonous strains as well as on strain robustness during sourdough back-slopping.

In the present study, the microbial ecology of nine wheat flour sourdoughs sampled in the Marche region was assessed in order to identify candidate yeast and LAB starter strains for the local baking industry. High loads of yeasts and LAB were seen in all of the sourdough samples analysed. These data are in agreement with those reported for wheat flour sourdoughs (DE VUYST and NEYSENS, 2005). The viable counts for the yeasts were lower than those recorded for the LAB for all of the sourdoughs under study.

With the restriction analysis of the 5.8S-ITS rRNA region, the results of this study fully confirm the usefulness of this analysis as a routine, fast technique for identifying yeasts from sourdough. However the published restriction pattern databases must be continuously updated. Indeed, new and atypical profiles can be generated by both reference and wild strains, as has been shown by the RFLP trials. The production of these atypical profiles could result from single mutations in the 5.8S-ITS region, which, in turn, are known to be responsible for the loss or gain of restriction sites. Accordingly, an intra-specific variability in the large subunit rDNA sequences of yeast species was previously seen with *C. lipolytica* (KURTZMAN and

ROBNETT, 1997) and *Clavispora lusitaniae* (LACHANCE *et al.*, 2003).

As far as the yeast ecology of our sourdough samples is concerned, *S. cerevisiae* was shown to dominate, and it was the only species detected in three out of four bakeries; namely, C, G and H. This finding confirms the high competitiveness of this species in wheat flour sourdough ecosystems (ROCHA and MALCATA, 1999; SUCCI *et al.*, 2003; PULVIRENTI *et al.*, 2004). Interestingly, in all four of the sourdoughs evaluated, the colony morphology of isolates of *S. cerevisiae* was different on WLN agar. According to CAVAZZA *et al.* (1992), this finding suggests the presence of a heterogeneous *S. cerevisiae* population. The presence of a biodiverse pool of *S. cerevisiae* strains among our isolates was confirmed by the visualization of different inter-delta amplification patterns. The δ1 and δ2 primers were previously used to successfully investigate the genetic polymorphism of yeasts from wheat flour sourdoughs (PULVIRENTI *et al.*, 2004). Interestingly, none of our yeast isolates showed fingerprints that were similar to those yielded by the *S. cerevisiae* strains isolated from the four compressed bakers' yeasts. This finding confirms the presence of a wild heterogeneous population of *S. cerevisiae* in the sourdough sample collected from bakery C, where the compressed bakers' yeast had never been used; at the same time, it suggests the exclusive presence of a wild *S. cerevisiae* community in the sourdough samples collected from bakeries A, G and H, where the compressed bakers' yeast was used in production lines different from those that were considered in the present study.

While *C. humilis* was previously identified as one of the prevalent species in wheat flour (GULLO *et al.*, 2003) and sourdoughs (FOSCHINO *et al.*, 2004), in the present study it was only isolated from one bakery. This suggests that this species was poorly adapted to the sourdough ecosystems analysed.

Similar to the RFLP, the restriction analysis of the amplified 16S rRNA gene proved to be a useful tool for identifying the sourdough LAB species. One of the main advantages of the techniques that rely on the restriction analysis of DNA sequences is that comprehensive restriction pattern databases can be built up and easily updated when new species, or even new profiles, are described. Given the availability of sequences of sufficient quality, the prediction of restriction patterns of deposited nucleotide sequences by applying computer-aided digestion helps to increase the discrimination power of these techniques.

Similar to other ecological investigations carried out on wheat sourdoughs (DE VUYST and NEYSENS, 2005), in the samples studied the LAB species with heterofermentative metabolism were prevalent. Remarkable differences in species richness were also found; in two sourdoughs considered (collected from bakeries C and E), only three species were isolated, while in the remaining samples, mixed LAB populations were detected that included up to six species. This high heterogeneity is probably due to the differences in the technological parameters (SALOVAARA, 1998) and/or to contamination from the raw materials and bakery environments (DE VUYST and NEYSENS, 2005).

Among the facultative heterofermentative species, *Lact. plantarum* was the most frequently isolated, while within the obligate heterofermentative group, *W. confusa* and *Lact. sanfranciscensis* prevailed; each was isolated from three sourdoughs. The frequent isolation of *Lact. plantarum* in wheat flour sourdoughs, as well as the association with *Lact. sanfranciscensis* and *Lact. brevis*, has been described (GOBBETTI *et al.*, 1994; GOBBETTI, 1998) and tentatively ascribed to the high adaptation of these micro-organisms to plant materials (e.g. maize and rye) (ROCHA and MALCATA, 1999).

Although type I sourdoughs are dominated by *Lact. sanfraciscensis* and *Lact. pontis* (VOGEL *et al.*, 1999) the composition of the microbial communities in the sourdoughs analyzed was closer to those usually detected in type-III sourdoughs, according to the classification proposed by VOGEL *et al.* (1999). A similar discrepancy was detected by RICCIARDI *et al.* (2005) in type-I sourdoughs used to manufacture Altamura bread in Apulia (southern Italy).

Based on the new classification of type-I sourdoughs proposed by DE VUYST and NEYSENS (2005), all of the sourdoughs analyzed in this study fall within the type-Ib group because they were prepared through multiple steps and characterized by extremely heterogeneous LAB populations, including species groups that were previously isolated from sourdoughs, i.e. *Lact. casei*, *Lact. plantarum*, *Lact. sakei*, *Lact. sanfranciscensis*, *Lact. gallinarum* (VOGEL *et al.*, 1999; EHRMANN and VOGEL, 2005). To our knowledge, representatives of the *Lact. salivarius* species have never been isolated from sourdoughs, but lactobacilli ascribed to *Lact. salivarius* were found in the samples collected from bakeries B and D.

The selection of micro-organisms with suitable functional properties and the establishment of optimized and controlled processing conditions in breadmaking are the key issues for the production of breads with the level and uniformity of quality currently required by the European consumer. Overall, the ecological investigations performed here have allowed both wild yeasts and LAB with baking potential to be found.

It is known that a good yeast strain for the sourdough process must possess definite properties, such as fast leavening of the dough and high amylolytic activity (LINKO *et al.*, 1997). With regard to the pool of *S. cerevisiae* isolates considered in this study, there was a high heterogeneity in both CO_2 production and amylolytic activity.

For the LAB, the high acid production during the first hours of the sourdough fermentation process is a desired trait of starter strains used in baking applications (CLARKE *et al.*, 2002). Based on this parameter, two isolates decreased the pH more rapidly, namely *Lact. plantarum* PB11 and *Lact. sakei* PB14, and are thus potential starter cultures.

Other microbial traits affect the stability of leavened baked goods, such as the type of organic acids produced during sourdough fermentation (DAESCHEL *et al.*, 1987). Lactic acid accounts for a more elastic gluten structure (LORENZ, 1983), while acetic acid positively affects the sensory properties of baked goods, and has anti-ropiness and anti-mould activities. To date, many efforts have been made to increase the concentration of acetic acid in sourdoughs, especially through the selection of starter strains ascribed to obligately heterofermentative species, like *Lact. sanfranciscensis* (GOBBETTI, 1998). When the amount of lactic acid and acetic acid produced by the isolates in this study were compared, the best performances were seen for *Lact. plantarum* PB11, PB46 and PB210, *Leuc. citreum* PB220 and *Lact. sanfranciscensis* PB211.

The amount of fermentable carbohydrates in flour largely depends on the extent of starch hydrolysis performed by the endogenous and microbial amylases and xylanases (MARTINEZ-ANAYA, 1996). Accordingly, a further criterion for selecting LAB starter strains is a high amylolytic ability, because this increases the availability of energy sources for non-amylolytic LAB and yeasts (SANNI *et al.*, 2002). Based on the results of the agar plate assays, the *Lact. plantarum* PB287 and *W. confusa* PB150 strains would provide the greatest hydrolysis of soluble starch.

The CO_2 released by the lactic acid bacteria has been reported to enhance

yeast metabolism, and consequently the yeast-leavening ability (GOBBETTI *et al.*, 1995). Accordingly, *Lact. plantarum* PB11 and PB 277, as well as *Lact. curvatus* PB115 could be candidates for starter strains, since they had the greatest gas production, together with *S. cerevisiae* PL97 and PL33. Although *Lact. plantarum* is a facultative heterofermentative species, which is not expected to produce high amounts of CO_2, under aerobic conditions, it can ferment lactate to acetate and CO_2 through a pathway which involves NAD+dependant and/or NAD+independent LDH, pyruvate oxidase and acetate kinase (MURPHY et al., 1985).

The overall results obtained in this study demonstrate the high biodiversity of the sourdough ecosystem and the effective possibility of exploiting this source of wild yeasts and LAB to find strains with baking potential. The yeast and LAB strains selected in the present study were included in a defined multispecies starter culture, which was used to prepare sourdoughs destined for in bread-making with barley flour (ZANNINI *et al.*, 2009).

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PAPER

CHEMICAL, NUTRITIONAL AND MICROBIOLOGICAL CHARACTERIZATION OF ORGANIC FONTINA PDO CHEESE

CARATTERIZZAZIONE CHIMICA, NUTRIZIONALE E MICROBIOLOGICA DEL FORMAGGIO FONTINA DOP BIOLOGICO

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ABSTRACT

Chemical, microbiological and nutritional characteristics of organic Fontina, an Italian Protected Designation of Origin (PDO) raw cow's milk cheese, and of bulk milk used for its production, were studied. Organic winter-indoor and summer-pasture productions were compared; organic summer samples were compared to conventional summer ones. The organic and conventional summer samples from fresh grass-fed cows showed better lipid-

RIASSUNTO

È stato condotto uno studio comparativo sulle caratteristiche chimiche e nutrizionali del formaggio Fontina DOP biologico prodotto durante i mesi di stabulazione e di alpeggio. Come conseguenza dell'alimentazione al pascolo delle bovine, i campioni di Fontina prodotti durante l'estate (sia biologici che convenzionali) così come i corrispondenti campioni di latte, hanno mostrato migliori caratteristiche nutrizionali: quantitativi più bassi di acidi gras-

- Key words: conjugated linoleic acid, fatty acid composition, Fontina PDO cheese, microbiological quality, organic dairy products, seasonal variation -

ic compositions than the winter ones. The hypercholesterolemic saturated fatty acids content was lower while the unsaturated fatty acids and rumenic acid contents were higher. No significant chemical and microbiological differences were observed between the organic and conventional summer milk and the cheese samples.

si saturi ipercolesterolemici e contemporaneamente valori più elevati di acidi grassi insaturi totali e di acido linoleico coniugato. Una seconda indagine è stata poi realizzata per verificare l'eventuale esistenza di differenze a livello chimico-microbiologico e nutrizionale tra i campioni di Fontina biologica e convenzionale prodotti durante l'estate. A tale riguardo non sono state rilevate differenze significative tra le due tipologie.

INTRODUCTION

Fontina cheese is a full fat semi-cooked cheese made with raw whole milk from a single milking exclusively from dual-purpose autochthonous Aosta cows (Red Pied, Black Pied and Chestnut). It is only produced locally in the mountain territory of the Autonomous Region of Aosta Valley (north-western Italian Alps) on a small-medium scale, but is well known and appreciated throughout Italy and in other parts of Europe. Since 1996 Fontina cheese has had the Protected Designation of Origin (PDO) denomination conferred by the European Union Commission (COMMISSION REGULATION, 1996). In 2003, the Manufacturing Rules were updated by the Region of Aosta Valley (in MATHIOU, 2007) and specific government financing fostered a new marketing phase with the production of organic Fontina PDO cheese at the Fromagerie Haute Val d'Ayas (Brusson, Aosta, Italy). This new product came about as a consequence of ethical concerns of consumers regarding both animal welfare and environmental issues such as soil acidification and eutrophication and greenhouse gas emissions. In particular, consumers are aware that organic foods have healthier and safer properties than traditional foods, and there has been increased interest and a growing demand for organic products, even though they are generally more expensive (MAGKOS *et al.*, 2006; WILLIAMS, 2002; ROSATI and AUMAITRE, 2004; HÄRING, 2003). Currently the Fromagerie Haute Val d'Ayas is the only cheese house that is allowed to produce organic Fontina PDO cheese in the entire Aosta Valley. About 1.3 million litres of cow milk are processed into organic Fontina cheese annually. Since 2003 about 13,000 forms have been produced according to the regulations for organic farming systems (COUNCIL REGULATION, 1991; COUNCIL REGULATION, 1999).

While organic Fontina is highly prized and consumed in great quantities, little is known about its chemical, microbiological and nutritional/nutraceutical features. Moreover, as the characteristics of traditional Fontina PDO cheese are known to vary widely between winter (hay/concentrate-fed cows) and summer (alpine pasture-fed cows) production (CHATEL *et al.*, 1995; CHATEL *et al.*, 1997), similar variations would be expected to occur in organic Fontina, but

no studies have been conducted to date to determine these variations.

Dietary factors are known to be the cause of the greatest variation in the amount of some important, biologically active fatty acids such as rumenic acid (*c*9*t*11 C18:2, the most abundant isomer of conjugated linoleic acid, to which the acronym CLA is referred in this study), in milk and dairy products (DHIMAN *et al.*, 2005). Milk fat obtained from cows grazing on alpine pastures has very high levels of CLA and omega-3 fatty acids (VAN DORLAND *et al.*, 2006). CLA has been shown to have biological activities that protect against carcinogenesis, obesity, diabetes, arteriosclerosis and other inflammatory diseases (COLLOMB *et al.*, 2006). Omega-3 fatty acids ensure brain structure and function and are negatively correlated with coronary heart diseases and psychiatric disorders (KRIS-ETHERTON *et al.*, 2003; BUCHER *et al.*, 2002; BOURRE, 2005).

Differences between winter- and summer-produced cheeses are mainly due to the chemical and microbiological characteristics of the milk. In the case of Fontina, better chemical characteristics of summer milk are combined with lower microbial contamination (CHATEL *et al.*, 1997), due to the particular conditions in which the cows are fed and milked in Aosta Valley (BARMAZ, 1992; CHATEL and BASSIGNANA, 2004). The lactic acid bacteria in summer milk frequently have a greater capacity to multiply and colonize than winter milk (AMBROSOLI and PISU, 1996). The summer milk also has a better aptitude for overcoming coliforms which therefore have less impact on cheese quality (CHATEL *et al.*, 1993-94; BARMAZ, 1995; CHATEL *et al.*, 1997). This factor is very important in the production of Fontina cheese that is typically characterized by a "low" acidifying activity of the lactic microflora involved (BATTISTOTTI *et al.*, 1976, 1977; MATHIOU, 2007), which has particular genetic features (BATTISTOTTI *et al.*, 1993; ANDRIGHETTO *et al.*, 2002) and is critical for attaining the typical sensorial characteristics of the final product.

Another area of interest involves the variation in quality between organic and conventional Fontina PDO cheese. To date there have been a limited number of studies to compare the chemical, nutritional and microbiological features of milk and dairy products obtained from organic and conventional farming systems. Moreover, the approach used in most of these studies (foods generally purchased from retail markets) could be affected by uncontrolled confounding factors (WILLIAMSON, 2007; BOURN and PRESCOTT, 2002). In general, organic products have a better fatty acid profile than conventional ones (more CLA and n3 fatty acids), but the results obtained by different authors are not always in agreement (BISIG *et al.*, 2007). BERGAMO *et al.* (2003) reported a marked difference between the fatty acid composition in organic and conventional Fontina cheese. This result could be quite misleading, because the difference could be related to winter production only. The differences in the chemical composition between organic and conventional Fontina cheeses should be greatly influenced by the season during which the milk is produced. Organic and conventional Fontina cheeses made from summer milk (cows have an almost identical pasture-based feeding regimen) are expected to have very similar gross composition and fatty acid profiles. From the microbiological point of view, mainly the safety aspects of organic *versus* conventional food have been evaluated; organic farming methods, while potentially liable to increase microbial contamination, do not have any higher risk of foodborne pathogens than conventional ones (MAGKOS *et al.*, 2003a; CENCIČ and BOREC, 2004; CHEN, 2005; MACHADO *et al.*, 2006). No conclusive information is available about microorganisms of technological interest in organic food.

The aims of this study were to: 1) determine if there are any differences in the chemical and nutritional attributes of organic Fontina PDO cheese made from winter- or summer-milk; 2) investigate whether organic and conventional Fontina PDO cheeses produced from summer milk can be distinguished based on their fatty acid composition and/or microbiological features; and 3) verify if the same trends observed in organic and conventional Fontina PDO cheeses also occur in the corresponding raw milk used to produce them. The influence that Fontina cheese processing has on the fatty acid profile was also evaluated, because no previous studies are available in the literature on this subject.

MATERIALS AND METHODS

Animal feeding and management

According to the latest Manufacturing Rules for Fontina PDO cheese (MATHIOU, 2007), cows were only fed with forage grown in the Aosta Valley territory during the entire experimental period (December 2006-August 2007).

From December to May, the cows (according to the organic farming system) were kept indoors on the farms located in the valley bottoms (800 to 1,000 m a.s.l.) and were fed the typical dietary regimen used for milk destined for the production of organic Fontina PDO cheese. The base diet consisted of an average of 70% hay and 30% concentrates; all feed was produced by organic agricultural methods, no chemical fertilizers or pesticides were used.

In June and July, cows (under conventional and organic farming systems) were allowed to pasture at medium altitudes (1,500-2,000 m a.s.l.), and then moved to higher altitudes (>2,000 m a.s.l.) where they stayed until the end of August. The traditional daily grazing regime allowed the cows to pasture for a total of six-seven h/day; they were moved indoors twice a day for milking (at 4:00 a.m. and 4:00 p.m.). For both organic and conventional systems, pasture feeding was characterized by *ad libitum* fresh grass plus a daily supplement of 1.5-2 kg of concentrate per head in order to meet nutritional requirements. The concentrate composition was in accordance with organic farming rules. The conventional system used a similar pasture-feeding regime but application of chemical fertilizers may have been used in the pasture lands.

Cheese-making

All Fontina PDO cheese samples were produced according to the Fontina PDO cheese Manufacturing Rules (MATHIOU, 2007). Briefly, milk from a single milking was collected in steel boilers, starters were added and the milk was coagulated with bovine rennet at 36°C (coagulation time 40 min). Starters were made up of a mixture of autochthonous strains of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *lactis* and *Lactococcus lactis* produced under the control of the Aosta Valley government. The curd was cut into maize grain-sized pieces and the curd-whey mixture was heated to 48°C under stirring. After 10 min rest in the whey, the curd was extracted and moulded in typical Fontina forms. Fontina PDO cheese has a typical flattened cylindrical form (35-45 cm diameter, 7-10 cm height and 7.5-12 kg weight).

The forms were subjected to moderate pressing for about 12 h and then transferred to the maturing site. The cheese matured for 3 months in natural caves (5°-12°C at >90% relative humidity). Cheeses were turned daily and the surfaces were manually salted and washed (*frottages*) with brine. During such period the typical red-brown rind smear is obtained.

Sampling

Milk and cheese samples were collected from the Fromagerie Haut Val d'Ayas (Brusson, Aosta, Italy). The organic bulk milk samples were collected monthly from December 2006 to August 2007; this time period covered the seasonal variation and was almost the entire lactation cycle of the dairy cows. In Aosta Valley calving commonly occurs in the autumn and winter months so that the cows are put out to pasture in the late lactation period when they have fewer nutritional requirements (CHATEL *et al.*, 1995; CHATEL *et al.*, 1997; MATHIOU, 2007). From December to May (stabled period) six samples (one sample/month) were taken and in June-August (summer pasture) six samples were collected (two samples/month, on consecutive days in order to have the same number of samples for all the groups for the statistical analysis). From June to August conventional bulk milk samples were also collected monthly (six samples), following the same schedule as that for summer organic samples. All milk samples were collected from the steel boilers just before the starters were added.

The batches of Fontina PDO cheeses corresponding to each of the collected milk samples were marked and ripened. A total of 18 forms were produced from the above-described milk batches (6 winter organic, 6 summer organic and 6 summer conventional). At the end of the three-month ripening period, a representative sample of each cheese batch was taken for the analyses. Cheese samples for microbiological analysis were only taken in the summer period.

Chemical analysis

Chemical and fatty acid composition of milk

One aliquot of each milk sample was stored in a portable refrigerator at 4°C and immediately transported to the laboratory to analyse the fat, protein, lactose and urea contents, and to determine the somatic cell count (SCC). A second aliquot to be used for the fatty acid analysis was stored for 4-5 h at 4°C before freezing at -20°C.

Milk fat, protein, lactose and urea contents were determined by infrared spectroscopy (MilkoScan FT6000, Foss Electric, Hillerød, Denmark) and the somatic cell count was carried out using an automatic cell counter (Fossomatic 5000, Foss Electric, Hillerød, Denmark).

For the fatty acid analysis, milk fat was extracted by centrifugation according to LUNA *et al.* (2005). The fatty acid methyl esters (FAME) were prepared by esterification with boron trifluoride as catalyst (AOAC, 2000) and determined by a gas chromatograph equipped with a HP88 capillary column (100 m x 0.25 mm ID, 0.2 m film thickness; J&W Scientific). The column temperature was held at 60°C for 1 min and then increased 20°C min^{-1} to a final temperature of 190°C, where it was held for 40 min. Temperatures of the injector and flame-ionization detector were maintained at 250° and 280°C, respectively; the injection volume was 0.1 μL; the nitrogen constant linear flow rate was set at 40 mL min^{-1}. Peaks were identified by comparing the retention times with pure CLA isomers (Matreya Inc., Pleasant Gap, PA, USA) and FAME (Restek Corporation, Bellefonte, PA, USA) standards. The fatty acid composition of the milk is expressed as the percentage of each fatty acid methyl ester per total FAMEs detected.

Chemical and fatty acid composition of cheese

Cheeses were cut into slices, the rind was removed and then the samples were frozen at -20°C until analysed. Dry matter (DM), ash, fat (EE) and protein determinations were performed according to AOAC (2000).

For the fatty acid analysis, after acid

hydrolysis of the sample, total lipids were extracted as reported by FOLCH *et al.* (1957). Esterification and fatty acid methyl esters separation, identification and quantification were carried out as described for milk.

All analyses were performed twice.

Microbiological analysis

Milk samples were collected in sterile 20-mL plastic screw-top containers, stored at 4°C in a portable refrigerator and immediately transported to the laboratory for the following analyses:

total aerobic mesophilic bacterial count on Petrifilm (3M) "Aerobic Count Plate" (72 h incubation at 30°C);

Coliforms and *Escherichia coli* on Petrifilm (3M) "*E. coli*/Coliform Count Plate" (24-48 h incubation at 37°C);

Proteolytics on Milk agar (Oxoid) (72 h incubation at 30°C).

For cheese analyses, the interior of each form was aseptically sampled in different points to obtain a final sample of approximately 100 g, which was submitted to the following determinations:

total aerobic mesophilic bacterial count on Petrifilm (3M) "Aerobic Count Plate" (72 h incubation at 30°C);

Coliforms and *Escherichia coli* on Petrifilm (3M) "*E. coli*/Coliform Count Plate" (24-48 h incubation at 37°C);

Yeasts on YGC agar (Oxoid) (5 days incubation at 25°C):

Streptococcus thermophilus on M17 agar (Oxoid) (24-48 h incubation at 45°C);

Lactobacillus spp. (thermophilic) on MRS agar (Oxoid) (48-72 h anaerobic incubation at 45°C);

Lactococcus spp. on M17 agar (Oxoid) (24 h incubation at 22°C).

All analyses were performed twice.

Statistical analysis

Based on the functional effects of individual fatty acids on cholesterol metabolism, the hypocholesterolaemic to hypercholesterolaemic fatty acids ratio (HH) was also assessed. Some changes were made in the mathematical equation reported by SANTOS-SILVA *et al.* (2002): 1. C20:4n6, C20:5n3, C22:5n3 and C22:6n3 were not considered in this study because they were not detected; 2. lauric acid was introduced in the denominator as a potential cholesterol-increasing agent (GERMAN and DILLARD, 2004); 3. myristic acid was multiplied by four because it has a greater influence as a hypercholesterolemic fatty acid than lauric and palmitic acids (GERMAN and DILLARD, 2004). The final formula was:

$$HH = (C18{:}1n9 + C18{:}2n6 + C18{:}3n3) \; / \; / \; (C12{:}0 + 4*C14{:}0 + C16{:}0).$$

The statistical analysis of the data was performed using the SPSS software (2004). The data (for both milk and cheese) were submitted to one-way analyses of variance (ANOVA) according to the following model:

$$X_{ij} = \mu + \alpha_i + \varepsilon_{ij},$$

where: X_{ij} = observation; μ = overall mean; α_i = effect of type of dairy product (1 = winter organic; 2 = summer organic; 3 = summer conventional); ε_{ij} = residual error.

The assumption of equal variances was assessed by Levene's homogeneity of variance test. If such an assumption did not hold, the Brown-Forsythe statistic was performed to test for the equality of group means instead of the F one (SPSS, 2004). Pairwise multiple comparisons were performed to test the difference between each pair of means (Tukey's HSD test and Tamhane's T2 in the cases of equal variances assumed or not assumed, respectively).

The effect of the cheese-making process on the fatty acid profile was assessed by performing a paired sample t-test. A

correlation analysis between fatty acids determined in Fontina cheese samples and in the corresponding unprocessed milk samples was also carried out.

Raw microbiological data were submitted to the independent sample t-test, in order to detect possible differences between organic and conventional samples.

Significance was declared at $P<0.05$. All data are expressed as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Chemical characteristics

Gross composition and somatic cell count

The gross composition of Fontina PDO cheese produced under organic (indoor and outdoor production) and conventional (outdoor production) farming systems is shown in Table 1. No statistically significant differences were found in dry matter, ash, protein and fat contents between organic cheese from winter/spring and summer productions. The organic and conventional samples from summer production also had very similar values. All cheese samples had >45% fat content (on the dry matter), as required by the Fontina Valle d'Aosta PDO Manufacturing Rules (in MATHIOU, 2007).

Concerning the corresponding milk samples, organic milk fat and protein percentages were significantly higher while lactose content was significantly lower in summer with respect to winter/spring months (Table 2). The stage of lactation is one of the main parameters that influences the gross composition of milk in ruminants. In the advanced lactation period, fat and protein concentrations normally increase, while lactose, even if it is the least variable component of milk, decreases. From June to August Aosta cows are normally in late lactation; so the results of this study reflect the trends that are generally observed as lactation progresses (AULDIST *et al.*, 1998). In summer, the gross composition of the organic and conventional milk samples was very similar, as already observed for the Fontina cheese samples. Such results are in agreement with the previous findings of PIRISI *et al.* (2002) and TOLEDO *et al.* (2002) on ewe and cow milk, respectively. TOLEDO *et al.* (2002) reported that their results were coherent because the gross composition of raw milk is mainly determined by a combination of the breed and diet (in their study as in this one organic and conventional milk samples were derived from the same cow breeds and the same diet). TOLEDO *et al.* (2002) also reported that the milk urea contents were ap-

Table 1 - Chemical composition of Fontina PDO cheese from organic (winter/spring and summer production) and conventional (summer production) farming systems[1].

	Organic (winter/spring) n = 6	Organic (summer) n = 6	Conventional (summer) n = 6	P value
DM (%)	60.90 ± 1.70	60.30 ± 0.36	61.17 ± 0.45	0.698
Ash (%DM)	7.45 ± 0.21	7.00 ± 0.76	6.57 ± 0.35	0.281
Protein (%DM)	38.90 ± 1.84	39.60 ± 0.70	38.63 ± 0.38	0.688
Ether extract (%DM)	46.40 ± 0.71	45.80 ± 1.30	47.20 ± 1.01	0.364

[1]Values are expressed as mean ± standard deviation of the mean.
DM = Dry Matter.

Table 2 - Chemical composition and somatic cell count of bulk milk used for the production of Fontina PDO cheese from organic (winter/spring and summer production) and conventional (summer production) farming systems[1].

	Organic (winter/spring) n = 6	Organic (summer) n = 6	Conventional (summer) n = 6	P value
Fat (%)	3.47 ± 0.14 b	3.70 ± 0.06 a	3.83 ± 0.09 a	0.004
Protein (%)	3.17 ± 0.08 b	3.36 ± 0.09 a	3.39 ± 0.04 a	0.004
Lactose (%)	4.89 ± 0.03 a	4.82 ± 0.02 b	4.78 ± 0.03 b	0.001
Urea (mg dL^{-1})	20.65 ± 2.06	21.35 ± 3.22	21.45 ± 4.66	0.916
SCC (n° 10^3 mL^{-1})	162 ± 34 b	265 ± 45 a	318 ± 56 a	0.001

[1]Values are expressed as mean ± standard deviation of the mean.
Different letters within rows indicate statistically significant difference among milk types.
SCC = Somatic Cell Count.

preciably lower in organic samples with respect to conventional samples. This was attributed to a difference in production intensity (forage/concentrate ratio). In the current study no statistically significant difference was found in the urea content, which reflects no discrepancy in the levels of milk production between the two farming systems in Aosta Valley.

Milk SCC is an important indicator of udder inflammation and mastitis infection in cows. In the organic milk samples, the value increased markedly from 162,000 mL^{-1} in winter to 265,000 mL^{-1} in summer. This increase normally occurs in late lactation (COULON *et al.*, 1996) and while grazing on alpine pastures (LAMARCHE *et al.*, 2000) and was previously observed for the same breeds by BIANCHI *et al.* (2003). Organic samples usually had a lower SCC than the conventional ones as already pointed out by OLIVO *et al.* (2005), TOLEDO *et al.* (2002) and HAMILTON (2000). Conventional summer milk samples exceeded the average value of 300,000 cells mL^{-1}. This value is commonly considered the threshold limit caused by management difficulties and unsanitary conditions of the cows (COULON *et al.*, 1998).

Fatty acid composition

Seasonal variations

The fatty acid profiles of Fontina cheese (winter organic, summer organic and summer conventional samples) are shown in Table 3. Organic Fontina PDO cheese obtained during the outdoor grazing period had a substantially higher nutritional value than organic Fontina produced during the stabled months. Almost all saturated fatty acids, with the exception of caproic (C6:0) and stearic (C18:0) acids, showed significantly lower values during the summer production. The percentage differences ranged from 12% in palmitic (C16:0) acid to 20% in heptadecanoic (C17:0) acid. The caprylic (C8:0), capric (C10:0) and lauric (C12:0) acid contents were approximately 17-20% lower in summer samples, while the variation in myristic (C14:0) acid was less (13%). Such results are important because lauric, myristic and palmitic acids are universally considered detrimental for human health due to their hypercholesterolemic activity, while caproic and stearic acids (the latter is a relatively abundant saturated fatty acid in dairy products) do not exert the same unfavourable effect (GERMAN and DILLARD, 2004).

Among the monounsaturated fatty acids, only the oleic acid (C18:1n9) content was higher in the organic summer samples; the extent of variation was however quite important (17%). Even if the other monounsaturated fatty acids did not show any change due to seasonal variation, it must be considered that oleic acid is the most abundant monounsaturated fatty acid in dairy products and is favorable from a nutritional perspective (HAUG *et al.*, 2007).

Along with the polyunsaturated fatty acids, linolenic acid (LNA) values were significantly higher in the organic summer samples compared to the organic indoor samples. The CLA levels were higher in the organic summer products (both milk and cheese), but in cheese samples the differences with respect to the indoor production were not statistically significant. Such results are in agreement with previous findings on the seasonal variation in the fatty acid profile of dairy products (REGO *et al.*, 2008; MOLKENTIN and GIESEMANN, 2007; LOCK and GARNSWORTHY, 2003; PRECHT and MOLKENTIN, 2000). The results are certainly due to the different feeding strategies in the two periods of analysis. In fact, pasture-fed ruminants were found to provide an improvement in the quality of the fatty acid composition of milk in several studies (D'URSO *et al.*, 2008; ATTI *et al.*, 2006; FERLAY *et al.*, 2006; WHITE *et al.*, 2001). Furthermore, alpine pasture-fed cows produce milk that has an extraordinarily relevant dietetic value for human health due to its high CLA and n3 fatty acid content (LEIBER *et al.*, 2005; HAUSWIRTH *et al.*, 2004). These positive qualities are probably due to the species-rich alpine meadows (with a high proportion of secondary plant ingredients) and to the animal energy deficiencies which lead to body fat mobilization. No significant difference related to seasonal variation was found in the linoleic acid (C18:2n6, LA) content of the cheese.

In general, the summer organic Fontina had a lower total SFA, and higher total MUFA and total PUFA contents and a higher HH ratio with respect to winter production.

Organic *versus* conventional (summer)

There were no significant statistical differences between the fatty acid profiles of summer organic and summer conventional Fontina cheese samples, or for the milk used for its manufacture, with respect to all the parameters determined (Tables 3 and 4). This is in contrast with the previous findings by BERGAMO *et al.* (2003) who reported higher LNA (more than 50%) and lower LA (about -31%) contents in organic Italian products (Fontina cheese included) with respect to conventional ones.

Concerning conjugated linoleic acid, earlier studies are in contrast with each other. Moreover, in the study by MOLKENTIN and GIESEMANN (2007) a seasonal influence on the variation of the CLA content in organic and conventional milk was observed where organic milk had a higher CLA content in the summer (but not the winter) months. In the current study, the CLA levels did not differ statistically between organic and conventional samples. These findings are in agreement with the results of ELLIS *et al.* (2006), TOLEDO *et al.* (2002) and BUTLER *et al.* (2008), but are in contrast with the results obtained by JAHREIS *et al.* (1996), BERGAMO *et al.* (2003) and COLLOMB *et al.* (2008).

It has been suggested that some parameters related to the fat composition could be used as markers for distinguishing organic dairy products from conventional ones (BERGAMO *et al.*, 2003; ELLIS *et al.*, 2006; MOLKENTIN and GIESEMANN, 2007). They are the CLA/LA ratio, the PUFA/MUFA ratio, the n3 fatty acids content (which were found to be significantly higher in organic products) and the n6/n3 fatty acids ratio (which in contrast was higher in the conventional ones). Our results show that the fatty

Table 3 - Mean values and standard deviations of fatty acid methyl esters (% of total FAMEs) in organic (winter/spring and summer production) and conventional (summer production) Fontina PDO cheese[1].

	Organic (winter/spring) n = 6	Organic (summer) n = 6	Conventional (summer) n = 6	P value
C6:0	1.25 ± 0.29	1.25 ± 0.13	0.97 ± 0.04	0.106
C8:0	0.93 ± 0.08 a	0.77 ± 0.03 b	0.63 ± 0.11 b	0.001
C10:0	2.46 ± 0.27 a	1.96 ± 0.22 b	1.62 ± 0.09 b	0.002
C10:1	0.20 ± 0.06	0.16 ± 0.01	0.15 ± 0.01	0.251
C12:0	3.28 ± 0.39 a	2.63 ± 0.23 b	2.22 ± 0.12 b	0.003
C12:1	0.23 ± 0.04	0.21 ± 0.02	0.20 ± 0.03	0.467
C14:0	12.84 ± 0.72 a	11.13 ± 0.19 b	9.86 ± 0.35 b	0.000
C14:1	0.43 ± 0.11	0.41 ± 0.04	0.44 ± 0.09	0.925
C15:0	0.75 ± 0.11	0.44 ± 0.04	0.58 ± 0.25	0.170
C15:1	1.32 ± 0.03	1.29 ± 0.16	1.26 ± 0.13	0.821
C16:0	33.95 ± 2.55 a	29.83 ± 0.65 b	28.20 ± 0.42 b	0.005
C16:1	0.36 ± 0.07	0.28 ± 0.09	0.31 ± 0.07	0.338
C17:0	1.71 ± 0.09 a	1.36 ± 0.16 b	1.46 ± 0.14 b	0.005
C17:1	0.66 ± 0.06	0.63 ± 0.03	0.61 ± 0.04	0.373
C18:0	11.10 ± 1.00	12.13 ± 0.35	11.97 ± 0.46	0.169
C18:1*c*9	25.11 ± 2.95 b	30.34 ± 0.47 a	33.89 ± 0.87 a	0.001
C18:2*c*9*c*12 (LA)	2.41 ± 0.38	2.52 ± 0.33	2.60 ± 0.47	0.785
C18:3*c*9*c*12*c*15 (LNA)	0.18 ± 0.03 b	0.26 ± 0.05 a	0.22 ± 0.02 ab	0.023
C18:2*c*9*t*11 (CLA)	1.25 ± 0.04 b	2.39 ± 0.70 ab	2.82 ± 0.23 a	0.046
CLA/LA	0.53 ± 0.13 b	0.98 ± 0.38 ab	1.12 ± 0.29 a	0.015
n6/n3	13.78 ± 3.78	9.96 ± 2.52	11.96 ± 2.90	0.312
SFA	67.89 ± 2.78 a	61.51 ± 1.12 b	57.52 ± 0.95 b	0.000
MUFA	28.30 ± 3.05 b	33.33 ± 0.60 a	36.86 ± 0.72 a	0.001
PUFA	3.84 ± 0.34 b	5.17 ± 0.53 a	5.64 ± 0.27 a	0.000
SFA/UFA	2.13 ± 0.25 a	1.60 ± 0.08 b	1.35 ± 0.05 b	0.001
PUFA/MUFA	0.14 ± 0.02	0.16 ± 0.01	0.15 ± 0.01	0.324
HH	0.32 ± 0.05 b	0.43 ± 0.10 a	0.52 ± 0.03 a	0.000

[1]Different letters within rows indicate statistically significant difference among Fontina PDO cheese types.
LA = Linoleic Acid; LNA = Linolenic Acid; CLA = Conjugated Linoleic Acid; SFA = Saturated Fatty Acids; MUFA = Monounsaturated Fatty Acids; PUFA = Polyunsaturated Fatty Acids; UFA = Unsaturated Fatty Acids; HH = Hypocholesterolemic to Hypercholesterolemic Fatty Acids ratio: HH = (C18:1n9 + C18:2n6 + C18:3n3) / (C12:0 + 4*C14:0 + C16:0).

acid analysis does not allow all organic and conventional dairy products to be clearly distinguished. In fact, both organic milk and Fontina cheese samples had the same PUFA/MUFA, CLA/LA and n6/n3 fatty acids ratios, as well as the same n3 fatty acid content, as conventional ones. The different feeding regimens in the two types of farming methods is the explanation (MAGKOS *et al.*, 2003b). In fact, organic farming systems are normally expected to provide larger amounts of fresh grass and less concentrates in the diet with respect to conventional ones, especially in the summer months (CROISSANT *et al.*, 2007). This was also true in Switzerland, even considering the noticeably high level of roughage (87%) normally used in Swiss mountain integrated farms (COLLOMB *et al.*, 2008). For this reason, many proposed analytical techniques for determining organic dairy products are based on parameters linked to pasture consumption. In

Table 4 - Mean values and standard deviations of fatty acid methyl esters (% of total FAMEs) in organic (winter/spring and summer production) and conventional (summer production) bulk milk used for the production of Fontina PDO cheese[1].

	Organic (winter/spring) n = 6	Organic (summer) n = 6	Conventional (summer) n = 6	P value
C6:0	1.16 ± 0.34	0.82 ± 0.54	0.79 ± 0.23	0.312
C8:0	0.89 ± 0.09 a	0.62 ± 0.06 b	0.58 ± 0.04 b	0.000
C10:0	2.34 ± 0.13 a	1.60 ± 0.13 b	1.64 ± 0.12 b	0.000
C10:1	0.27 ± 0.24	0.15 ± 0.03	0.13 ± 0.02	0.494
C12:0	3.00 ± 0.33 a	2.31 ± 0.09 b	2.34 ± 0.19 b	0.005
C12:1	0.22 ± 0.06	0.20 ± 0.05	0.20 ± 0.03	0.818
C14:0	12.43 ± 0.78 a	10.05 ± 0.05 b	10.01 ± 0.46 b	0.000
C14:1	0.37 ± 0.02 b	0.62 ± 0.07 a	0.61 ± 0.07 a	0.000
C15:0	0.58 ± 0.10 b	0.78 ± 0.06 a	0.78 ± 0.05 a	0.006
C15:1	1.24 ± 0.19	1.34 ± 0.16	1.29 ± 0.12	0.707
C16:0	34.12 ± 2.37 a	28.49 ± 0.68 b	28.43 ± 0.96 b	0.002
C16:1	0.36 ± 0.07	0.46 ± 0.08	0.46 ± 0.11	0.178
C17:0	1.83 ± 0.04	1.72 ± 0.46	1.76 ± 0.10	0.868
C17:1	0.69 ± 0.06	0.69 ± 0.09	0.67 ± 0.08	0.915
C18:0	11.88 ± 1.08	13.18 ± 0.60	13.33 ± 0.35	0.065
C18:1*c*9	25.10 ± 2.37 b	32.39 ± 0.43 a	32.44 ± 1.20 a	0.000
C18:2*c*9*c*12 (LA)	2.01 ± 0.10	2.20 ± 0.29	2.34 ± 0.06	0.198
C18:3*c*9*c*12*c*15 (LNA)	0.20 ± 0.04	0.23 ± 0.07	0.22 ± 0.02	0.633
C18:2*c*9*t*11 (CLA)	1.33 ± 0.09 b	2.25 ± 0.23 a	1.98 ± 0.15 a	0.000
CLA/LA	0.66 ± 0.03 b	1.04 ± 0.19 a	0.85 ± 0.04 ab	0.011
n6/n3	10.32 ± 2.32	9.94 ± 1.96	10.54 ± 1.19	0.935
SFA	68.21 ± 2.29 a	59.57 ± 0.89 b	59.67 ± 1.30 b	0.000
MUFA	28.26 ± 2.42 b	35.76 ± 0.50 a	35.80 ± 1.48 a	0.000
PUFA	3.53 ± 0.20 b	4.68 ± 0.38 a	4.54 ± 0.20 a	0.000
SFA/UFA	2.16 ± 0.21 a	1.47 ± 0.05 b	1.48 ± 0.08 b	0.000
PUFA/MUFA	0.13 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	0.861
HH	0.32 ± 0.05 b	0.49 ± 0.02 a	0.49 ± 0.04 a	0.000

[1]Different letters within rows indicate statistically significant difference among milk types.
LA = Linoleic Acid; LNA = Linolenic Acid; CLA = Conjugated Linoleic Acid; SFA = Saturated Fatty Acids; MUFA = Monounsaturated Fatty Acids; PUFA = Polyunsaturated Fatty Acids; UFA = Unsaturated Fatty Acids; HH = Hypocholesterolemic to Hypercholesterolemic Fatty Acids ratio: HH = (C18:1n9 + C18:2n6 + C18:3n3) / (C12:0 + 4*C14:0 + C16:0).

summer, both organic and conventional Fontina PDO cheeses are produced with milk derived from the same cow breeds grazing on very similar alpine pastures (climatic conditions, altitude and local flora) in a limited area of production. In contrast to the findings of COLLOMB *et al.* (2008), no differences were found in the proportion of grasses fed to cows in the two mountain farming systems (fresh grass from pasture *ad libitum* in both cases). The amount of concentrate supplementation was also comparable (about 2 kg *versus* 1.5 kg head^{-1} day^{-1} in organic and conventional farms, respectively). The slightly higher amounts of concentrates commonly used in the organic farms is, however, unusual because the use of concentrates is usually limited in organic farming systems although it can be justified for commercial reasons (added value of organic milk and cheese). The aboe-mentioned similarities in the diet do not allow organic dairy

products to be distinguished on the basis of their fat composition.

Further investigations will be needed to determine if the above-mentioned markers are suitable for identifying organic and conventional Fontina PDO cheeses produced under indoor-stabled conditions and which markers can be used to differentiate organic from conventional Fontina cheese produced in the outdoor grazing months. Parameters correlated with fertilization (stable nitrogen isotopes) could be appropriate for the latter purpose. In fact, the ^{15}N content is higher in organic manure than in artificial fertilizers, thus affecting the relative $\delta^{15}N$ content in plants. In the organic farming systems the use of artificial fertilizers is prohibited; consequently, as previously pointed out by MOLKENTIN and GIESEMANN (2007), $\delta^{15}N$ may differ between organically and conventionally produced feed. Such differences could be reflected in the milk protein.

Effect of the cheese-making process

The majority of studies dealing with the influence of processing conditions on the fatty acid composition of dairy products have mainly related to variations in the CLA amounts only. Although results obtained from different studies are quite contrasting, the normal processing procedures for dairy products (such as fermentation steps, heat treatments, storage and ripening) have only a negligible effect on the CLA concentrations (BISIG *et al.*, 2007). In the current study, no statistically significant differences were observed in the CLA amounts between Fontina cheese and unprocessed raw milk. A strong correlation between the CLA content of milk and cheese was observed ($P<0.0001$). Such results are consistent with the earlier findings by LUNA *et al.* (2007) on Spanish cow and ewe PDO cheeses, and by NUDDA *et al.* (2005) on sheep Pecorino Romano and Ricotta cheeses.

To the best of our knowledge, only one previous study has dealt with the effect of milk composition on fatty acids other than CLA (LUCAS *et al.*, 2005). In general, Fontina cheese samples had significantly higher levels of lauric, myristic and linoleic acids and significantly lower levels of palmitoleic, heptadecanoic, heptadecenoic and stearic acids with respect to the corresponding milk ones (Table 5). Milk and Fontina cheese samples showed a statistically significant correlation for the majority of parameters determined. Similar to the results reported by LUCAS *et al.* (2005), the results of the present study show that caprylic, decanoic, lauric, myristic, palmitic, stearic and oleic acids, as well as CLA, LNA, total SFA, MUFA and PUFA were significantly correlated between the cheese samples and the corresponding unprocessed milk ones. In contrast to LUCAS *et al.* (2005), no statistically significant correlations were found for caproic and linoleic acids. In our study, none of the other fatty acids (decenoic, myristoleic, pentadecanoic, pentadecenoic, palmitoleic and heptadecanoic acids) were significantly correlated in milk and cheese samples. This latter group of fatty acids was not considered in the study by LUCAS *et al.* (2005). Further investigations will be needed to evaluate the influence of specific processing steps on the variation of individual fatty acids in milk and dairy products.

Microbiological characteristics

Organic *versus* conventional (summer)

Bulk milk had low microbial contamination (data not shown). The mesophilic bacterial counts for both organic and conventional systems were always below the legal limit for raw milk cheeses ($5{,}00 \times 10^4$ CFU mL^{-1}), and the coliforms and proteolytics never exceeded a few hundred CFU mL^{-1}. No significant differences were found between the two systems and between the winter and summer productions, although

Table 5 - Effect of the Fontina cheese-making process on fatty acid methyl esters (% of total FAMEs)[1].

	Milk n = 18	Cheese n = 18	P value	Pearson's correlation coefficient	Sig.
C6:0	0.98 ± 0.38	1.18 ± 0.24	0.064	0.522	0.082
C8:0	0.74 ± 0.16	0.82 ± 0.15	0.053	0.712	0.009
C10:0	1.98 ± 0.39	2.13 ± 0.43	0.069	0.809	0.001
C10:1	0.21 ± 0.18	0.18 ± 0.05	0.609	-0.399	0.198
C12:0	2.66 ± 0.43	2.85 ± 0.55	0.012	0.933	0.000
C12:1	0.21 ± 0.05	0.21 ± 0.03	0.714	0.666	0.018
C14:0	11.23 ± 1.37	11.68 ± 1.40	0.020	0.919	0.000
C14:1	0.49 ± 0.14	0.43 ± 0.09	0.199	-0.054	0.866
C15:0	0.68 ± 0.13	0.63 ± 0.19	0.538	-0.520	0.083
C15:1	1.28 ± 0.16	1.30 ± 0.09	0.710	0.366	0.242
C16:0	31.29 ± 3.40	31.49 ± 3.18	0.487	0.961	0.000
C16:1	0.41 ± 0.09	0.33 ± 0.08	0.019	0.263	0.408
C17:0	1.79 ± 0.06	1.56 ± 0.06	0.005	0.412	0.183
C17:1	0.68 ± 0.06	0.64 ± 0.05	0.015	0.597	0.040
C18:0	12.57 ± 1.06	11.58 ± 0.88	0.000	0.956	0.000
C18:1*c*9	28.73 ± 4.15	28.61 ± 4.39	0.780	0.939	0.000
C18:2*c*9*c*12 (LA)	2.14 ± 0.21	2.49 ± 0.36	0.009	0.193	0.548
C18:3*c*9*c*12*c*15 (LNA)	0.21 ± 0.04	0.21 ± 0.05	0.682	0.703	0.011
C18:2*c*9*t*11 (CLA)	2.47 ± 0.60	2.65 ± 1.41	0.506	0.929	0.000
CLA/LA	1.15 ± 0.24	1.09 ± 0.60	0.660	0.621	0.031
n6/n3	10.28 ± 1.86	12.37 ± 3.45	0.040	0.445	0.147
SFA	63.92 ± 4.79	63.70 ± 5.02	0.681	0.935	0.000
MUFA	32.02 ± 4.31	31.70 ± 4.32	0.505	0.928	0.000
PUFA	4.07 ± 0.61	4.62 ± 0.90	0.000	0.956	0.000
SFA/UFA	1.82 ± 0.39	1.80 ± 0.39	0.629	0.960	0.000
PUFA/MUFA	0.13 ± 0.01	0.15 ± 0.02	0.000	0.907	0.000
HH	0.40 ± 0.10	0.40 ± 0.10	0.530	0.908	0.000

[1]Values are expressed as mean ± standard deviation of the mean.
LA = Linoleic Acid; LNA = Linolenic Acid; CLA = Conjugated Linoleic Acid; SFA = Saturated Fatty Acids; MUFA = Monounsaturated Fatty Acids; PUFA = Polyunsaturated Fatty Acids; UFA = Unsaturated Fatty Acids; HH = Hypocholesterolemic to Hypercholesterolemic Fatty Acids ratio: HH = (C18:1n9 + C18:2n6 + C18:3n3) / (C12:0 + 4*C14:0 + C16:0).

the latter usually had slightly higher counts. *Escherichia coli* was never detected. Such favourable hygienic reports are most likely correlated with the rapid processing of the milk (according to Manufacturing Rules) i.e. within 2 h of milking, when it still has natural antibiotic properties.

No significant microbiological differences were found between the summer cheeses from the two systems, due to the great variability among samples, as indicated by the high SD values (Table 6). The total lactic microflora at the end of the maturing period was abundant in both cases, but was slightly higher (about $1.3x10^8$ *versus* $8.0x10^7$ CFU g^{-1}) in conventional Fontina. In this case, *Streptococcus thermophilus* was the prevalent component (almost $1.0x10^8$ CFU g^{-1}), while in the organic system samples *S. thermophilus* was replaced by the mesophilic *Lactococcus* spp. group (where the starter species *Lactococcus lactis* is likely to be found). The thermophilic *Lactobacillus* spp. group

Table 6 - Microbial counts (CFU/g*10^3) in organic and conventional summer Fontina PDO cheeses[1].

Microbial Groups	Organic n = 6	Conventional n = 6	P value
Streptococcus thermophilus	$5.30x10^4 \pm 5.23x10^4$	$9.53x10^5 \pm 1.34x10^3$	0.516
Lactococcus spp.	$2.25x10^3 \pm 2.48x10^3$	450 ± 354	0.489
Thermophilic *Lactobacillus* spp.	$2.55x10^4 \pm 3.46x10^4$	$3.30x10^4 \pm 2.55x10^4$	0.830
Total Coliforms	77.45 ± 103	60.20 ± 84.57	0.872
Escherichia coli	0.51 ± 0.70	0.21 ± 0.28	0.654
Total Mesophilic Aerobic Count	$2.15x10^4 \pm 2.12x10^3$	$2.15x10^4 \pm 3.04x10^4$	1.000
Yeasts	11.50 ± 3.54	177.50 ± 243.95	0.512

[1]Values are expressed as mean ± standard deviation of the mean.

(where the other starter species *Lactobacillus delbrueckii* subsp. *lactis* belongs) was present in similar quantities (2.5-$3.0x10^7$ CFU g^{-1}) in both cheese types. Considering the very low contamination levels in the bulk milk, such high levels of the lactic microflora could be related to the use of starters at the beginning of processing.

At the end of the maturing period the coliform count was quite low (<$1.0x10^5$ CFU g^{-1}) in both organic and conventional Fontina. As desired in Fontina manufacturing, the coliforms were surpassed by the lactic acid microflora. *E. coli* were sporadically found, in very low numbers (<$1x10^3$ CFU g^{-1}).

The uniform microbial situation found in this study for both organic and conventional Fontina could have been due to the low number of cheese forms that were analyzed. It is quite probable that the starter used (the same for conventional and organic cheeses) played an important role in integrating the initially low autochthonous population and standardizing its subsequent evolution. It must be recalled that the starter is a mixture of locally isolated species that have particular genetic features (ANDRIGHETTO *et al.*, 2002) that have been specifically chosen to obtain a correct and consistent maturation.

CONCLUSIONS

This study provides the first characterization of the chemical, microbiological and nutritional quality of organic Fontina PDO cheese comparing the cheeses produced during the indoor period and the summer-pasture period; the organic and conventional Fontina cheeses produced in summer were also compared. As for the cheeses produced in the winter months, those produced from June to August had a higher HH ratio and higher total PUFA content (in both milk and cheese samples); the CLA (milk samples) and LNA (cheese samples) levels were also higher in the summer productions. These findings are consistent with a significant decrease in the SFA/UFA ratio and in the amount of total SFA observed in both the summer milk and cheese samples. Highly significant correlations were found between the main groups of fatty acids (SFA, MUFA and PUFA) as well as between parameters commonly used as nutritional indicators (SFA/UFA and HH) in milk and Fontina cheese. The results clearly show that the Fontina cheese produced in summer has better nutritional characteristics due to the beneficial effect of the pasture-based diet on milk fat composition.

As in previous reports (MAGKOS *et al.*, 2006), the results of this study did not

attribute superior safety or nutritional quality to organic foods of animal origin with respect to conventional ones. It seems that the feeding regimen strongly affects the future nutritional value and quality of ruminant-derived products (BUTLER *et al.*, 2008; GIVENS, 2005). This assumption is confirmed by the fact that 1) no variation was found between organic and conventional milk and Fontina cheese samples during the grazing months due to an almost identical diet for the cows and 2) summer conventional milk and cheese samples had a better lipid fraction value than the winter organic ones.

Every country has different regulations for organic production, and each typical PDO cheese is produced according to particular rules that can lead to differences in the organic and conventional systems with respect to chemical and microbiological quality. This variation is also strongly affected by the season of production. Pasture-derived alpine dairy products (and not only the organic ones) often meet the requirements for organic production and have extraordinary nutritional/nutraceutical and physiological properties. For these reasons they should be recognized as "organic foods" regardless of the livestock farming system used.

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PAPER

ANTIOXIDANT COMPOSITION OF TOMATO PRODUCTS TYPICALLY CONSUMED IN ITALY

COMPOSIZIONE IN ANTIOSSIDANTI DI CONSERVE DI POMODORO TRADIZIONALMENTE CONSUMATE IN ITALIA

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ABSTRACT

Several commercial samples of tomato pulp and tomato puree produced by major Italian companies and corresponding to different lots were examined to determine the overall composition and concentrations of antioxidant components (namely *all-trans*-lycopene, β-carotene, ascorbic acid, total polyphenols and rutin), in order to obtain reliable nutritional information about tomato preserves that are typically consumed in Italy. Analysis

RIASSUNTO

L'obiettivo del lavoro è stato di valutare la composizione in generale e, in particolare, la concentrazione dei composti antiossidanti (*trans*-licopene, β-carotene, acido ascorbico, polifenoli totali e rutina) di una serie di campioni commerciali di polpe e passate di pomodoro prodotti dai maggiori marchi aziendali italiani, allo scopo di ottenere informazioni nutrizionali affidabili sui derivati del pomodoro maggiormente consumati in Italia.

- Key words: ascorbic acid, carotenoids, polyphenols, rutin, tomato pulp, tomato puree -

of the data showed that the two types of products differ in ascorbic acid and rutin concentrations, whereas similar values were found for lycopene, β-carotene and total phenolics. The differences can be ascribed to the quality of the raw materials as well as the processing technology used, as shown by the pilot-scale experimentation. The compositional data reported can be used to improve and enhance nutritional information about Italian tomato products.

L'analisi dei risultati ha evidenziato che le due tipologie di prodotto differiscono per il contenuto di acido ascorbico e di rutina, mentre presentano valori simili di licopene, β-carotene e polifenoli totali. Queste differenze possono essere imputate, in base ai risultati della sperimentazione su scala pilota, alla diversa qualità delle materie prime e al processo di produzione. I dati di composizione ottenuti, infine, possono essere utilizzati per migliorare e completare le informazioni nutrizionali sui derivati del pomodoro italiano.

INTRODUCTION

The Mediterranean diet is associated with a low incidence of chronic diseases and, in particular, of several types of cancer (RISO *et al.*, 2003). The health properties of this dietary model are ascribed to the high content of complex carbohydrates, fibers and antioxidants and to a low animal fat content. For this reason, nutritional recommendations include greater consumption of fruit and vegetables that are rich in antioxidants such as vitamin C and E, carotenoids and phenolic substances.

Tomato is a typical dietary component in southern Europe and is known to be a rich source of antioxidants, mainly carotenoids, ascorbic acid and polyphenols (ABUSHITA *et al.*, 1997; LEONARDI *et al.*, 2000; BEECHER, 1998; GIOVANELLI *et al.*, 1999; STEWART *et al.*, 2000; FRUSCIANTE *et al.*, 2007). Over the last few decades scientists have investigated the disease-preventing properties of tomatoes and tomato components. They have demonstrated that regular tomato consumption is associated with a reduced risk of various types of cancer (RAO and AGARWAL, 2000) and in particular prostate cancer (GIOVANNUCCI, 1999) and colorectal adenomas (ERHARDT *et al.*, 2003). Tomato consumption has also been correlated with a lower incidence of cardiovascular disease (WILLCOX *et al.*, 2003; PANDEY *et al.*, 1995; RAO and AGARWAL, 2000).

Several studies have been carried out on the effects of processing and thermal treatments on tomato antioxidants and antioxidant activity (NGUYEN and SCHWARTZ, 1999; GIOVANELLI *et al.*, 2001; ZANONI *et al.*, 2003; SAHLIN *et al.*, 2004; CHARANJEET *et al.*, 2004). The results have shown substantial lycopene stability and variable changes in ascorbic acid and polyphenols. These studies have been conducted on different products, cultivated in different countries and obtained from different tomato varieties, so it is difficult to compare the nutritional data. Moreover, data are often given on a fresh weight basis, whereas tomato products are marketed at different concentration levels, depending on local food legislation and traditions.

The aim of this study was to evaluate

the antioxidant composition of tomato products that are mainly consumed in Italy, where both fresh and processed tomato products are very popular.

In 2003 in Italy 5.2 million tons of tomatoes were processed into tomato concentrate (16%), whole peeled tomatoes (38%), tomato pulp (29%) and tomato puree (14%) (ISMEA, 2004). These products are exclusively made of tomato; only NaCl and citric acid are added (the latter to adjust the pH).

Domestic consumption of tomato products in Italy is mainly based on tomato puree, whole peeled tomatoes and tomato pulp, which make up the principal ingredients of sauces for pasta, pizza and various meat and fish recipes. Tomato concentrate is principally used in industrial food production and catering (ISMEA, 2004).

For the purpose of this study, 15 tomato purees (passata) and 13 tomato pulps (diced tomato in tomato juice) from three major national companies were purchased on the market and the usual quality parameters and the antioxidant composition were determined, particularly the quantities of all-*trans*-lycopene and all-*trans*-β-carotene, ascorbic acid, total phenolics and rutin (quercetin-3-rutinoside). Furosine [ε-N-(2-furoyl-methyl-L-lysine)] was determined as a heat-damage index in the tomato products.

Two experimental tomato purees and two experimental tomato pulps were produced using a pilot plant and then analysed for the same parameters as the commercial samples, in order to obtain some information about the effects of industrial processing on the antioxidant composition of the products.

Data are given on both fresh weight and dry weight basis, in order to compare products with different solid contents. These data could be of great interest to nutritionists and can be used to enhance and complete nutritional food tables.

MATERIALS AND METHODS

Tomato products

Tomato puree (700 g glass bottles) and tomato pulp (450 g cans) were purchased on the market during the study period (approximately 6 months). Three major Italian brands were chosen and various production lots from each producer were collected. The samples are identified in the text by X (pulp) or Y (puree), a letter (*a, b, c*) for the producer and a number (1 to 6) for the lot.

The two experimental tomato purees and two experimental tomato pulps were produced on a pilot scale, using an industrial variety of tomato (Brigade) and a high-lycopene experimental hybrid (PC 30956), characterised by high-pigment genes (SM, sp^+, hp-2, u^+); the latter will be referred to as the high-lycopene tomato. Both raw materials were supplied by Cirio Ricerche, Piana di Monte Verna, Salerno, Italy. Experimental samples are identified by letters *d* and *e*, which correspond to the different tomato varieties (Brigade and high-lycopene, respectively).

For pilot-scale production, about 100 kg of tomatoes were washed in a soak tank with mechanical agitation, put on a roller conveyor where the tomatoes were rinsed by spraying with water. The pulp production plant was a continuous line, consisting of a heater where the tomatoes were dipped in hot water (100°C) for 30 s, put into a hopper where tomatoes were cooled by a cold-water spray. The tomatoes were then conveyed to a scoring knife which made a shallow slit in the peel of each tomato and next through jaws that gripped the peels and slipped the tomatoes out of the peels. The tomatoes then moved onto rotating rubber rollers that eliminated any residual peels. Defective tomatoes were eliminated by an operator. Tomatoes were then mechanically sliced (12 mm width); the slices were drained on vibrating sieves to remove se-

rum and seeds, and then passed through a chopper (to obtain 12 mm cubes). Cans (400 g) were manually filled with tomato pulp (310-320 g) and tomato juice at 10.5-11.0°Bx (80-90 g) and sterilised by heating in a 100°C water bath to obtain a treatment equal to F_{100} = 12. The sterilizing effect was monitored by a thermocouple inserted into one of the cans. After sterilisation, the cans were cooled by immersion in tap water.

Tomato puree was produced by a discontinuous line; the washed tomatoes were crushed and blanched in the same plant, by pushing tomatoes through a grid into a tubular heat exchanger where the product was heated up to 96°-98°C. The product was directly discharged into a refiner made of two subsequent vertical tubular sieves (1.5 mm and 0.8 mm, respectively); the product was forced through the sieves by a rotating tree. The refined puree passed through the sieves while the peels, seeds and coarse material were discarded. The tomato puree was then concentrated to the desired degree (9°Brix) in a batch pan evaporator (capacity 50 L) for 35-40 min at 65°C (- 600 mm Hg). The desired amount of NaCl was added (about 0.5%), then the tomato puree was heated up to 92°C in the same pan before manual hot-filling of the glass bottles (750 g). The bottles were sterilised in a water bath (100°C) to obtain the desired heat treatment (F_{100} = 12-14), then cooled in the same bath with tap water.

Analytical methods

Analytical determinations were carried out on tomato pulp, puree and on raw tomatoes. For the analysis of raw tomatoes, 5-6 tomatoes were blanched in boiling water for 30 s and manually peeled. The peeled tomatoes were homogenised in an ice bath (60 s with a Waring Blender at moderate speed) and aliquots were immediately extracted with a suitable solvent. For the analysis of commercial samples, two packages of each sample were poured together and homogenised with the Waring Blender for a few seconds.

Dry weight was determined gravimetrically after drying in a vacuum oven at 70°C (AOAC, 2002); NaCl, pH, titratable acidity and reducing sugars were determined as reported by PORRETTA (1991).

Colour indices (L*, a* and b*) were measured with a tristimulus chromameter (Minolta, Tokyo, Japan, model CR-210), calibrated with a red standard (No. 482, Bureau Communitaire de Reference: L* = 25.6, a* = 33.5, b* = 14.7) and red colour index is expressed as a*/b*; each colour value was obtained by 5 repeated measurements.

Ascorbic acid was extracted with 0.3% meta-phosphoric acid and determined by HPLC and electrochemical detection, as previously described (GIOVANELLI *et al.*, 2002). All-*trans*-lycopene and all-*trans*-β-carotene were determined by extraction with tetrahydrofuran and subsequent reverse-phase HPLC analysis with UV detection, as previously described (GIOVANELLI *et al.*, 2002). Total phenolics were extracted, purified by separation on a C18 Sep Pak cartridge (Waters, Millford, MA, USA) and determined by Folin-Ciocalteau reagent, while rutin (quercetin-3-rutinoside) was determined by reverse-phase HPLC analysis on the phenolic extract (GIOVANELLI *et al.*, 2001); furosine was determined by HPLC after acid hydrolysis and is expressed as milligrams of furosine per 100 g of protein, as reported by HIDALGO and POMPEI (2000).

Statistical analysis

All chemical analyses were carried out in triplicate and data are expressed as the mean value ± standard deviation. The data were submitted to analysis of variance (one-way ANOVA), F-test and Multiple Range Test (LSD), performed by Statgraphics plus 5.1 package (Graphics Software Systems, Rockville, MD, USA).

Principal Components Analysis (PCA) was also performed in order to interpret differences between the tomato products; the Unscrambler 9.5.0 software was used (Camo As, Trondheim, Norway).

RESULTS

Table 1 reports the analytical composition of the raw tomatoes used for pilot-scale production. Two lots of *d* tomatoes (Brigade) and three lots of *e* tomatoes (high-lycopene hybrid) were analysed during the experimental period. The *e* tomatoes were higher in solid content and richer in lycopene, β-carotene, ascorbic acid and total polyphenols than the *d* tomatoes, while there were no substantial differences in rutin concentration (data compared on a dry weight basis). Both varieties had higher concentrations of all the antioxidant components when compared to the literature data (DAVIES and HOBSON, 1981; ABUSHITA *et al.*, 1997; LEONARDI *et al.*, 2000). LENUCCI *et al.* (2006) reported higher concentrations of lycopene and total phenolics in high-pigment tomato hybrids.

The analytical data for the commercial tomato pulp (13 lots) and tomato puree (15 lots) are shown in Tables 2 and 3, respectively. The mean values for each analytical parameter in the commercial products and data obtained for the two experimental tomato pulps and purees are also reported.

Commercial tomato pulps

With regard to tomato pulp (Table 2), the typical composition of the product showed a certain variability. The dry matter varied between 6.82 and 9.18 and the °Brix between 5.43 and 7.70. The NaCl varied from 1.13 to 5.50 g/kg.

The pH varied within a narrow range (4.19-4.38), as did the titratable acidity (5.15-6.60). The reducing sugar content was between 26.39 and 34.5 g/kg, with

Table 1 - Analytical data of raw tomatoes used for experimental pulp and puree production on fresh and dry weight basis (mean value, standard deviation in brackets).

	Dry weight (g/100 g)	°Brix	pH	a*/b*	lycopene (mg/kg)		β-carotene (mg/kg)		ascorbic acid (mg/kg)		total phenolics (mg/kg)		rutin (mg/kg)	
					fw	dw	fw	dw	fw	dw	fw	dw	fw	dw
raw tomatoes:														
tomato *d*	5.64	4.55	4.35	2.30	129	2295	2.8	50	183	3245	253	4487	23	400
(n=2)	(0.35)	(0.07)	(0.07)	(0.01)	(12)	(213)	(0.1)	(3)	(27)	(502)	(15)	(270)	(4)	(70)
tomato *e*	6.50	5.07	4.47	2.71	187	2870	5.5	85	272	4180	329	5068	23	351
(n=3)	(0.57)	(0.68)	(0.07)	(0.11)	(15)	(231)	(0.8)	(13)	(46)	(708)	(23)	(355)	(3.4)	(52)

n = number of lots examined;
fw = fresh weight basis;
dw = dry weight basis.

Table 2 - Analytical data of commercial and experimental tomato pulps expressed on fresh weight (mean value, standard deviation in brackets).

	Dry weight (g/100 g)	°Brix	NaCl (g/kg)	pH	Total acidity (g/kg)	Reducing sugars (g/kg)	a*/b*	Lycopene (mg/kg)	β-carotene (mg/kg)	Ascorbic acid (mg/kg)	Total phenolics (mg/kg)	Rutin (mg/kg)	Furosine (mg/100 g protein)
commercial pulps:													
X*a*1	6.82	5.43	1.33	4.27	5.71	28.04	1.95	138.2	3.92	162.9	223	14.7	53.9
	(0.04)	(0.06)	(0.06)	(0.01)	(0.14)	(0.05)	(0.025)	(0.8)	(0.02) (2.9)	(7.1)	(0.4)	(4.0)	
X*a*2	7.12	6.20	1.37	4.26	6.42	31.27	2.00	146.8	4.01	87.7	244	13.4	56.6
	(0.08)	(0.01)	(0.06)	(0.01)	(0.07)	(0.14)	(0.006)	(3.1)	(0.04)	(0.6)	(1.3)	(0.2)	(6.5)
X*a*3	8.11	7.13	3.77	4.28	5.66	34.36	2.05	145.7	2.29	119.9	277	17.4	77.2
	(0.08)	(0.06)	(0.06)	(0.01)	(0.06)	(0.25)	(0.015)	(0.5)	(0.02)	(3.2)	(12.7)	(0.6)	(3.4)
X*a*4	8.56	7.70	4.83	4.22	5.19	31.93	1.97	127.5	6.99	104.3	344	32.1	79.7
	(0.05)	(0.01)	(0.06)	(0.01)	(0.13)	(0.34)	(0.015)	(4.0)	(0.56)	(0.4)	(2.1)	(0.1)	(4.0)
X*a*5	8.10	6.97	5.03	4.22	5.24	30.91	1.98	114.2	4.01	67.7	350	30.5	58.5
	(0.10)	(0.06)	(0.06)	(0.01)	(0.03)	(0.73)	(0.006)	(2.7)	(0.56)	(3.8)	(5.9)	(0.6)	(1.0)
X*a*6	8.27	7.03	5.50	4.24	5.41	29.47	1.94	106.3	3.30	106.4	279	17.3	51.0
	(0.06)	(0.15)	(0.04)	(0.02)	(0.57)	(0.68)	(0.010)	(6.7)	(0.76)	(5.7)	(2.4)	(0.7)	(6.7)
X*b*1	7.97	6.40	1.53	4.32	5.58	34.44	2.01	140.9	3.73	124.1	355	24.3	57.3
	(0.02)	(0.10)	(0.06)	(0.01)	(0.46)	(0.05)	(0.020)	(9.2)	(0.01)	(2.0)	(5.2)	(0.2)	(3.2)
X*b*2	7.77	6.33	1.80	4.38	5.57	32.17	1.76	186.3	3.48	111.8	350	19.4	55.0
	(0.02)	(0.06)	(0.01)	(0.01)	(0.05)	(0.08)	(0.031)	(11.1)	(0.16)	(2.3)	(3.2)	(0.7)	(4.3)
X*b*3	9.18	6.77	1.30	4.34	6.23	40.58	2.04	187.3	3.88	133.7	373	23.7	65.2
	(0.07)	(0.06)	(0.10)	(0.01)	(0.02)	(1.21)	(0.006)	(18.8)	(0.36)	(1.5)	(3.2)	(0.1)	(1.9)
X*b*4	8.00	6.80	1.13	4.33	6.22	30.25	2.00	144.7	4.51	127.6	472	21.4	53.8
	(0.10)	(0.10)	(0.06)	(0.01)	(0.03)	(1.90)	(0.010)	(2.3)	(0.30)	(6.4)	(28.4)	(0.9)	(1.3)
X*c*1	8.33	7.17	2.50	4.21	6.16	34.44	2.05	162.8	2.93	104.3	352	19.3	70.4
	(0.11)	(0.06)	(0.30)	(0.01)	(0.08)	(1.49)	(0.025)	(8.0)	(0.4)	(3.3)	(3.4)	(0.7)	(1.0)
X*c*2	7.76	6.23	3.97	4.19	6.60	26.39	2.01	163.5	4.08	66.4	390	17.6	54.8
	(0.01)	(0.06)	(0.06)	(0.01)	(0.05)	(0.37)	(0.020)	(6.4)	(0.05)	(1.1)	(22.8)	(0.1)	(2.3)
X*c*3	8.57	7.23	3.17	4.26	5.15	32.99	2.06	148.6	6.79	79.9	621	37.5	54.8
	(0.06)	(0.06)	(0.21)	(0.01)	(0.23)	(0.25)	(0.006)	(0.2)	(0.22)	(1.8)	(6.6)	(0.7)	(2.4)
mean value	*8.04*	*6.72*	*2.86*	*4.27*	*5.78*	*32.10*	*1.99*	*147.1*	*4.15*	*107.4*	*356*	*22.2*	*60.6*
	(0.61)	*(0.59)*	*(1.60)*	*(0.06)*	*(0.49)*	*(3.54)*	*(0.078)*	*(24.0)*	*(1.34)*	*(27.5)*	*(103.2)*	*(7.2)*	*(9.4)*
experimental pulps:													
X*d*	8.28	7.17	2.93	4.30	5.92	30.91	2.06	162.1	4.12	207.1	200	12.6	119.3
	(0.07)	(0.06)	(0.07)	(0.05)	(0.08)	(0.59)	(0.006)	(5.3)	(1.63)	(3.4)	(4.0)	(0.4)	(3.1)
X*e*	7.54	7.20	3.17	4.26	5.57	27.37	2.14	229.6	7.93	255.9	309	13.1	70.0
	(0.07)	(0.01)	(0.06)	(0.04)	(0.09)	(0.47)	(0.006)	(6.5)	(0.20)	(2.2)	(2.0)	(0.2)	(3.0)

Table 3 - Analytical data of commercial and experimental tomato purees expressed on fresh weight (mean value, standard deviation in brackets).

	Dry weight (g/100 g)	°Brix	NaCl (g/kg)	pH	Total acidity (g/kg)	Reducing sugars (g/kg)	a*/b*	Lycopene (mg/kg)	β-carotene (mg/kg)	Ascorbic acid (mg/kg)	Total phenolics	Rutin (mg/kg)	Furosine (mg/100 g protein)
commercial purees:													
Ya1	8.78	7.53	8.07	4.39	5.58	34.35	2.07	148.2	5.09	219.5	452	52.3	58.3
	(0.03)	(0.06)	(0.06)	(0.01)	(0.02)	(0.40)	(0.006)	(12.9)	(0.03)	(4.5)	(4.3)	(0.26)	(4.5)
Ya2	8.32	7.33	7.80	4.40	5.02	32.87	1.97	127.4	4.85	208.6	432	49.5	62.3
	(0.03)	(0.06)	(0.01)	(0.01)	(0.04)	(0.27)	(0.006)	(6.4)	(0.09)	(2.6)	(2.2)	(0.43)	(1.1)
Ya3	8.68	7.87	7.53	4.35	5.90	33.45	1.99	139.8	5.58	173.9	483	55.6	74.6
	(0.01)	(0.06)	(0.06)	(0.01)	(0.01)	(1.40)	(0.006)	(16.2)	(0.14)	(3.0)	(9.7)	(0.38)	(1.1)
Ya4	9.13	8.2	3.26	4.30	5.43	38.13	2.01	168.2	5.30	227.0	375	59.2	120.0
	(0.10)	(0.10)	(0.12)	(0.01)	(0.10)	(1.77)	(0.030)	(0.2)	(0.85)	(3.4)	(7.4)	(0.70)	(5.0)
Ya5	9.39	8.27	4.60	4.41	6.58	40.61	2.03	196.3	5.39	191.7	342	59.1	87.6
	(0.02)	(0.12)	(0.10)	(0.04)	(0.26)	(3.26)	(0.025)	(3.0)	(0.54)	(6.9)	(10.0)	(1.44)	(3.5)
Ya6	8.52	7.53	5.60	4.40	5.42	32.66	2.01	145.0	4.93	183.8	381	49.6	96.6
	(0.02)	(0.15)	(0.36)	(0.04)	(0.11)	(0.34)	(0.015)	(1.4)	(0.87)	(12.3)	(8.5)	(2.13)	(5.1)
Yb1	9.49	8.47	5.37	4.43	5.63	38.89	2.17	172.6	4.54	196.3	385	40.6	66.9
	(0.04)	(0.06)	(0.06)	(0.01)	(0.04)	(0.27)	(0.006)	(4.2)	(0.39)	(4.3)	(0.4)	(0.48)	(2.3)
Yb2	9.33	8.3	5.43	4.38	6.16	36.52	2.13	166.5	5.62	240.9	399	37.3	59.8
	(0.06)	(0.10)	(0.21)	(0.02)	(0.06)	(0.42)	(0.015)	(14.0)	(0.31)	(6.6)	(6.8)	(1.01)	(2.2)
Yb3	9.78	8.83	5.07	4.41	6.12	38.44	2.14	195.7	5.69	259.5	381	36.9	52.8
	(0.02)	(0.15)	(0.06)	(0.02)	(0.05)	(0.05)	(0.015)	(5.6)	(0.45)	(7.0)	(14.6)	(1.32)	(4.9)
Yb4	9.60	8.7	5.23	4.41	5.72	37.99	2.16	193.9	5.65	229.3	413	39.5	59.8
	(0.20)	(0.10)	(0.06)	(0.02)	(0.22)	(1.17)	(0.010)	(0.7)	(0.44)	(2.7)	(8.4)	(0.51)	(3.3)
Yb5	9.60	8.83	5.77	4.43	6.08	36.06	2.15	191.6	6.58	205.1	518	33.8	45.6
	(0.10)	(0.15)	(0.31)	(0.02)	(0.11)	(0.82)	(0.020)	(5.8)	(0.04)	(1.2)	(17.6)	(2.23)	(2.3)
Yc1	8.38	7.33	5.17	4.32	6.59	35.02	1.97	190.6	4.57	70.3	388	51.2	86.3
	(0.04)	(0.06)	(0.06)	(0.01)	(0.18)	(0.78)	(0.006)	(4.0)	(0.05)	(0.7)	(15.5)	(1.90)	(1.9)
Yc2	8.49	7.43	4.67	4.35	5.32	35.88	2.10	157.2	4.71	184.0	226	50.1	92.3
	(0.01)	(0.06)	(0.06)	(0.01)	(0.15)	(0.29)	(0.015)	(1.0)	(0.03)	(8.4)	(8.3)	(2.93)	(6.7)
Yc3	9.03	8.1	4.53	4.25	6.36	34.66	2.08	183.6	4.46	128.2	446	55.5	71.7
	(0.06)	(0.10)	(0.06)	(0.01)	(0.16)	(1.44)	(0.020)	(10.8)	(0.20)	(1.7)	(14.6)	(0.29)	(6.1)
Yc4	8.83	7.83	4.67	4.30	6.10	36.42	2.01	146.2	5.31	108.7	417	58.1	74.6
	(0.06)	(0.06)	(0.06)	(0.05)	(0.12)	(0.52)	(0.015)	(8.6)	(0.55)	(0.8)	(27.5)	(2.50)	(0.5)
mean value	9.02	8.04	5.52	4.37	5.87	36.13	2.07	168.2	5.22	188.5	403	48.6	73.9
	(0.49)	(0.53)	(1.33)	(0.05)	(0.47)	(2.35)	(0.07)	(23.2)	(0.57)	(51.3)	(66.7)	(8.72)	(19.6)
experimental purees:													
Yd	8.87	8.47	4.33	4.37	6.72	32.69	2.28	149.1	4.46	205.1	306	28.7	26.9
	(0.06)	(0.06)	(0.06)	(0.04)	(0.10)	(0.52)	(0.006)	(2.5)	(0.56)	(2.1)	(4.7)	(3.25)	(1.7)
Ye	9.02	7.93	4.07	4.42	6.66	34.38	2.43	230.6	8.51	389.7	327	36.5	172.0
	(0.10)	(0.06)	(0.06)	(0.04)	(0.14)	(0.51)	(0.006)	(0.6)	(0.36)	(1.9)	(6.6)	(1.78)	(8.9)

the exception of one sample that had a much higher value (40.58 mg/kg). The colour index a*/b* was quite homogeneous, with values between 1.76 and 2.06.

With regard to the antioxidant components, the lycopene content, i.e. the main carotenoid found in tomatoes, varied between 106.3 and 187.3 mg/kg, whereas the β-carotene concentrations were much lower (2.29-6.99 mg/kg). The ascorbic acid concentration varied between 66.4 and 162.9 mg/kg.

Significant variability was observed in the total polyphenols (between 223-621 mg/kg). Rutin, the most representative flavonoid in tomatoes (STEWART *et al.*, 2000) and especially important for its high antioxidant activity, showed high variability (13.4-37.5 mg/kg), which was not strictly related to the total polyphenol concentration.

The furosine content is directly related to the intensity of the heat treatment and represents a heat damage index (HIDALGO and POMPEI, 2000). The furosine values were substantially homogeneous (between 51.0 and 79.7 mg/100 g protein), and reflect a substantial standardization of the industrial processing technologies.

Experimental pulps

The data regarding the general composition of the experimental pulps (dry matter, °Brix, pH, total acidity, reducing sugars and a*/b*), were within the ranges of the commercial products.

The pulp samples obtained with the pilot plant from the two tomato varieties used in this study (X*d* and X*e*) had much higher ascorbic acid concentration values than those found in the commercial products. The lycopene concentration was also higher than that determined in the commercial pulps. The X*e* sample had very high concentrations of lycopene and ascorbic acid, which are typical of the raw material (Table 1). On the contrary, the total polyphenol and rutin values of the experimental pulps were similar to the lower values detected in the commercial products. The furosine level of the experimental pulps was higher than that of the commercial pulps, especially for sample X*d*. This was probably due to the sterilization treatment performed in a static bath at 100°C, which was more severe than that applied at the industrial level.

Commercial tomato purees

Analysis of the commercial purees (Table 3) shows that the dry matter value was higher than in the pulps with an average of 9 g/100 g (between 8.32 and 9.78 g/100 g), and the °Brix value ranged between 7.33 and 8.83 (average value 8.04). The NaCl content was higher than in the pulps and varied between 3.26 and 8.07 g/kg.

The pH value of the pulp samples was homogeneous (average value 4.37) as was the titratable acidity (average value 5.87 g/kg), which is similar to that observed in the pulps. The reducing sugar values varied a little, between 32.87 and 40.61 g/kg, with an average value slightly higher than that of the pulps.

The colour index (a*/b*) values of the purees were very similar, with an average value of 2.07. The lycopene content of the commercial purees ranged between 127.4 and 196.3 mg/kg, while the β-carotene content varied between 4.54 and 6.58 mg/kg (average values of 168.2 and 5.22 mg/kg, respectively). These data were similar to those obtained for the pulps. Ascorbic acid concentration values showed some variability (between 108.7 and 259.5 mg/kg, average value 188.5 mg/kg) and were generally higher than those in commercial pulps.

The total polyphenol and rutin values were higher than those observed in the pulps and had less variability. The average furosine concentration was 73.9 mg/100 g protein and the spread was relatively low; sample Y*a*4 was an ex-

ception with a much higher concentration (120 mg/100 g protein).

Experimental purees

The compositional data of the experimental purees Y*d* and Y*e* were within the variability range observed in the commercial products with the exception of the NaCl concentration that was slightly lower, the total acidity which was slightly higher and the colour index which was significantly higher.

The pilot plant purees had high lycopene concentrations, similar to those found in the corresponding pulps; a particularly high value was recorded in sample Y*e* obtained from the high-lycopene tomato variety. The β-carotene content was similar to that of the experimental pulps and was within the range observed in the commercial purees.

The concentration of ascorbic acid was very high in sample Y*e*, as already observed in the pulps. The total polyphenol values were slightly higher than those obtained in the corresponding pulps; the concentrations were lower than those found in the commercial products. Rutin values were lower than those found in the commercial purees and were significantly higher than those of the commercial pulps. The furosine values for samples Y*d* and Y*e* were 26.9 and 172.0 mg/100 g protein, respectively. This marked difference was presumably due to a prolonged heat treatment of sample Y*e*.

Statistical evaluation

To analyse the variability of the parameters considered, the data were submitted to one-way ANOVA and multiple range test using the LSD (Least Significant Difference) procedure; the samples were grouped by brand and product type (pulp and purees).

In comparing products with different concentrations of solids, all the data refer to the dry weight (Table 4). The analysis was conducted on the parameters of greater nutritional significance: NaCl, reducing sugars and the antioxidant components. The results show a significant effect ($p<0.001$) between groups for all the parameters, except for reducing sugars ($p<0.05$) and β-carotene.

The NaCl concentration was higher in the purees than in the pulps, and varied significantly according to the brand. The pulps and purees of brand *a* had a higher NaCl content than those in brands *b*

Table 4 - Comparison of different brands of commercial tomato pulp and puree (data expressed on dry weight).

	NaCl (g/kg dw)	Reducing sugars (g/kg dw)	Lycopene (mg/kg dw)	β-carotene (mg/kg dw)	Ascorbic acid (mg/kg dw)	Total phenolics (mg/kg dw)	Rutin (mg/kgdw)
products:							
X*a* (n=6)	45.0 bc	396.5 ab	1673 a	52.0 a	1404 b	3628 a	262.5 a
X*b* (n=4)	17.7 a	416.9 c	2004 c	47.6 a	1512 b	4735 b	270.3 a
X*c* (n=3)	39.4 b	379.6 a	1932 bc	55.7 a	1014 a	5499 c	298.7 a
Y*a* (n=6)	70.5 e	400.9 bc	1744 ab	59.0 a	2283 c	4687 b	615.5 c
Y*b* (n=5)	56.2 d	393.1 ab	1924 c	58.7 a	2366 c	4385 b	393.9 b
Y*c* (n=4)	54.9 cd	409.1 bc	1953 c	54.8 a	1414 b	4238 b	618.4 c
F ratio	20.68	3.04	4.75	2.00	34.17	8.18	127.6
p	***	*	***	ns	***	***	***

n = number of lots examined;
n.s. = not significant; * p<0.05; ** p<0.01; *** p<0.001.

and *c*. The reducing sugars showed lower variability and no significant differences were noted between brands or type of product. The lycopene concentration appeared to be influenced by the brand but not by the type of product: the pulps and purees of brands *b* and *c* had very similar lycopene contents which were significantly higher than that of brand *a*.

The ascorbic acid concentration was influenced by the product and the brand. The pulps and purees of producers *a* and *b* had significantly higher ascorbic acid contents than those of brand *c*, with generally higher concentrations in the purees. The total polyphenol values were significantly different with respect to the brands only for the pulps. The rutin concentrations were similar in the pulps from different brands whereas there were differences in the purees according to the brands; the rutin concentration was significantly higher in the purees than in the pulps.

Data regarding the composition of each product were then submitted to principal component analysis (PCA) in order to study the influence of the various parameters. As it can be seen in Fig. 1 (score plot), the first two principal components explain 58% of the total variance. The tomato samples are well separated in the space. The tomato pulp samples are separated from tomato purees along the first component (explained variance 41%). The second component (explained variance 17%) distinguishes sample Y*e* (experimental sample particularly high in lycopene, β-carotene and ascorbic acid) from the other products.

Fig. 2 (Bi-plot) shows how the loading plot (descriptors) is superimposed on the mapping of the samples (Fig. 1, score plot). As can be seen, pulp samples on the right of the first component are characterised by lower scores of all the parameters considered. On the contrary, the puree samples are richer in polyphenols, NaCl, reducing sugars, rutin and dry matter and have higher pH and °Brix values. Along the second dimension, the X*e*, and in particular the Y*e* samples, differ from the others due to the high lycopene, ascorbic acid, colour index and furosine values. These values are dependent on the raw material used and on the sterilisation process applied.

DISCUSSION AND CONCLUSION

The data obtained in this study show that, although there was some variability in the composition of commercial tomato products, the nutritional values were quite homogeneous, especially when considered on a dry weight basis. With respect to the concentration of reducing sugars, all products ranged between 379 and 409 g/kg dw, regardless of the kind of product and the brand; the same was observed for lycopene (ranging from 1,673 and 2,004 mg/kg dw) and β-carotene (from 47.6 to 59 mg/kg dw). Some differences between the samples were found in the NaCl content, which was higher in the tomato purees in order to meet sensory requirements; ascorbic acid also seemed to be higher in tomato puree, which could have been due to the different industrial stabilisation treatments applied to these products. During this study, time-temperature process profiles were examined for the industrial lines of tomato pulp and tomato puree (the evaluation was carried out in a factory of producer *a*); the sterilisation treatment of tomato pulp was carried out at 118°C for 30 min, whereas the sterilisation treatment of tomato puree was carried out at 92°C for 10 min. This difference is due to the fact that, in the case of pulp, the core of the solid pieces in the can must reach the final temperature, while in tomato puree, the product is rapidly heated to the sterilisation temperature in a heat exchanger and then hot-filled. The holding time and entire treatment time are therefore much shorter. Various studies have shown that ascorbic acid can be degraded during to-

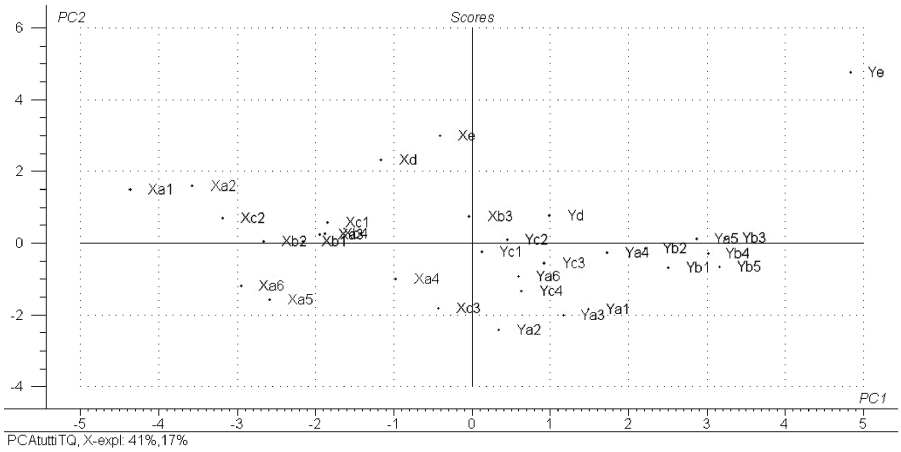


Fig. 1 - Principal Component score plot from analytical parameters of tomato pulp (X) and puree (Y) samples.

mato processing, depending on the severity of the heat treatment (ABUSHITA *et al.*, 2000; GIOVANELLI *et al.*, 2001; ZANONI *et al.*, 2003; SAHLIN *et al.*, 2004). Lower ascorbic acid concentrations in tomato pulp samples can be ascribed, at least in part, to the above-described differences in the sterilisation process.

With regard to polyphenols, significant differences in rutin concentrations were observed between pulps and purees. Rutin (quercetin-3-rutinoside) is the most representative flavonoid in tomatoes and it has been shown that flavonoids are located mainly in the skins (STEWART *et al.*, 2000). The higher rutin concentration in tomato puree can again be attributed to the technology used. The tomatoes are peeled before processing to give tomato pulp, while tomato puree is obtained by crushing and refining whole tomatoes, with a subsequent higher extraction from the peels. The rutin concentrations found in commercial Italian products were consistent with those reported by STEWART *et al.* (2000) for tomato puree. The PCA analysis confirmed these observations. The purees could be distinguished from the pulps by a higher solid concentration (which means higher values for reducing sugars, °Brix and solid content). These results correspond to somewhat higher con-

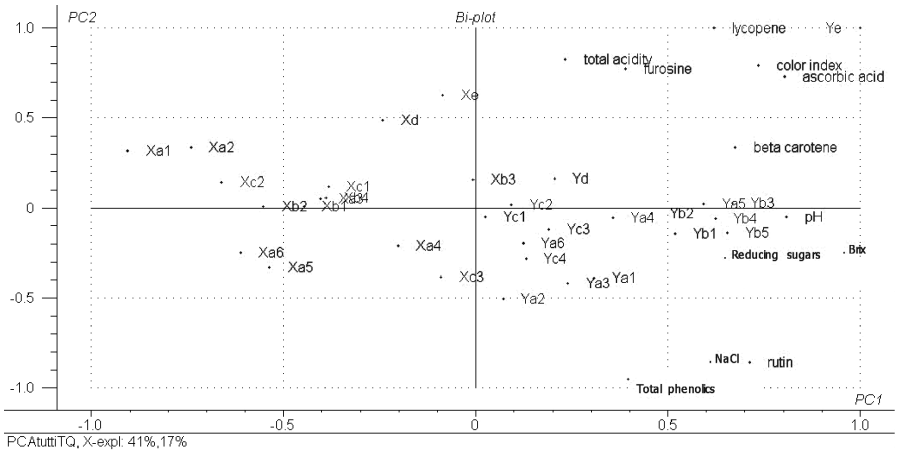


Fig. 2 - Principal Component Bi-plot: loading plot superimposed over score plot from analytical parameters of tomato pulp (X) and puree (Y) samples.

centrations in antioxidant components (lycopene, β-carotene, ascorbic acid, total polyphenols and rutin).

In general, the levels of antioxidant substances in pulps and purees obtained from the pilot-scale production were correlated with corresponding raw materials, as reported in Table 1. These results show that industrial processing preserves, in part, the nutritional characteristics of the raw material.

It can be concluded that the carotenoid composition of Italian tomato pulps and purees, which are the main forms of domestic consumption of tomato preserves, varies over a relatively narrow range, especially when considered on a dry weight basis, and depends mostly on the antioxidant concentration of the raw material. Tomato purees generally have a higher ascorbic acid and rutin concentration, depending upon the technological process adopted. These data can be used to compile a more complete nutritional characterisation of Italian tomato preserves.

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PAPER

FATTY ACID, MINERAL AND PROXIMATE COMPOSITION OF SOME SEAWEEDS FROM THE NORTHEASTERN MEDITERRANEAN COAST

ACIDI GRASSI, SOSTANZE MINERALI E COMPOSIZIONE ELEMENTARE DI ALCUNE ALGHE DELLA COSTA DEL NORD-EST MEDITERRANEO

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ABSTRACT

Proximate analysis, fatty acid profile and mineral content were investigated in brown (*Cystoseira corniculata*, *Padina pavonia)* and red seaweeds (*Laurencia papillosa* and *Jania rubens*). The highest protein content was obtained for *P. pavonia* (35.40% dry weight) followed by *L. papillosa* (34.65% dry wt). Lipid contents of seaweeds ranged from 6.73% for *L. papillosa* to 0.12% dry weight for *J. rubens*. The fatty acid compositions of seaweed species includ-

RIASSUNTO

Sono stati studiati la composizione elementare, il profilo in acidi grassi ed il contenuto in sostanze minerali di alghe brune (*Cystoseira corniculata*, *Padina pavonia)* e rosse (*Laurencia papillosa* and *Jania rubens*). Il più alto contenuto proteico è stato ottenuto per la *P. pavonia* (35,40% peso secco) seguita dalla *L. papillosa* (34,65% peso secco). Il contenuto lipidico delle alghe variava dal 6,73% per la *L. papillosa* allo 0,12% (peso secco) per la *J. rubens*. La

- Key words: Fatty acids, mineral content, proximate analysis, seaweeds -

ed 31.66-47.97% saturated fatty acids (SFA), 23.04-32.06% monounsaturated fatty acids (MUFAs) and 11.58-18.08% polyunsaturated fatty acids (PUFAs). Mineral analysis showed that the seaweeds contain high amounts of both macro minerals (Ca, Mg, Na, P and K) and trace elements (Zn and Mn).

composizione in acidi grassi delle specie algali evidenziava dal 31,66 al 47,97% di acidi grassi saturi (SFA), dal 23,04 al 32,06% di acidi grassi monoinsaturi (MUFAs) e dall'11,58 al 18,08% di acidi grassi polinsaturi (PUFAs). La determinazione delle sostanze minerali mostrava che le alghe contenevano alte quantità sia di macro minerali (Ca, Mg, Na, P and K) sia di oligoelementi (Zn and Mn).

INTRODUCTION

Macroalgae, also known as seaweeds, are quite diverse and included members of the red, brown and green algae. Fossil evidence suggests that coastal marine seaweed communities have existed for about 500 million years (GRAHAM and WILCOX, 2000). They make up an important biomass in coastal ecosystems. In coastal habitats they are the primary producers in the food chain, producing organic material from sunlight, carbon dioxide and water (LEE, 1989). The primary productivity of seaweed communities is equal to or greater than that of the most productive terrestrial plant communities (GRAHAM and WILCOX, 2000). In addition to their role as primary producers, they provide an environment that is favourable for other living organisms and for reproduction (WILSON, 2002; FLEURENCE, 1999).

Some seaweeds, especially brown and red seaweeds, are harvested and used as food or for commercial products (LEE, 1989; SZE, 1998). Seaweeds are rich in minerals, vitamins and some specific components. The mineral content of some seaweeds accounts for up to 36% of the dry weight (BURTIN, 2003). The nutrient composition of seaweed varies depending on species, geographical area, season and water temperature. Written records confirm that humans have harvested seaweed for more than 2000 years. The annual world production of seaweed was 12.9 tonnes in 2002 (FAO, 2003). Human consumption of seaweed is widespread in the Pacific and in Asian countries, where several seaweed species are commercially farmed (SZE, 1998). In Europe, seaweeds are used to produce additives (e.g. alginates) or meal for animal nutrition. In most cases, seaweed is used in human food or animal feed for their mineral content and/or for the functional properties of their polysaccharides (FLEURENCE, 1999; BURTIN, 2003). Today, seaweed polysaccharides are used in the food, cosmetics, paint, crop, paper and building industries (DERE *et al.*, 2003).

The chemical content of seaweed can be assessed by using advanced analytical techniques. The results have indicated new opportunities for their use in different technological applications.

Turkey is surrounded on three sides by seas, so there is an enormous seaweed biomass available for use. However, seaweed harvesting is very rare in Turkey. A number of studies have been carried out on the taxonomy and distribution of seaweed on the northeastern Mediterranean coast of Turkey (AYSEL

and GÜNER, 1980; DURAL *et al.*, 1989; AYSEL and GEZERLER-ŞIPAL, 1996; ÖZTÜRK *et al.*, 1996) and the chemical content of seaweeds in the Turkish seas have been analyzed by several researchers (ÇETINGÜL and GÜNER, 1996; BILGIN and ERTAN, 2002; DERE *et al.*, 2003).

The aim of this study was to determine the chemical constituents (proximate composition, fatty acid profiles and macro and trace mineral elements) of some seaweed species collected from the northeastern Mediterranean coast of Turkey. Four species (*C. corniculata*, *L. papillosa, J. rubens, P. pavonia*), common in the region, were selected for the study.

MATERIALS AND METHODS

Sample collection

Seaweeds were collected from the northeastern Mediterranean coast of Turkey in October 2005. Four species, commonly distributed in the region, were selected; *Jania rubens* (L.) Lamouroux and *Laurencia papillosa* (Agardh Greville) belong to Rhodophyta (red seaweeds), while *Cystoseira corniculata* (Turner) Zanardini and *Padina pavonia* (L.) Gaillon belong to Phaeophyta (brown seaweeds). Epiphytes were removed and the samples were then washed with sea water and distilled water. The samples were then packaged in polyethylene bags and stored at -20°C until chemical analyses were performed.

Proximate composition

The seaweed samples were analyzed in triplicate for proximate composition: lipid content by the BLIGH and DYER (1959) method, moisture content by the AOAC (1990) method, total crude protein by the Kjeldahl method (protein conversion factor: 6.25) (AOAC, 1984), ash content by the AOAC (1990) method and neutral detergent fibre content as described by VAN SOEST *et al.* (1991) using an ANKOM Fibre Analyzer.

For lipid analyses, approximately 10 g of sample were homogenized with a chloroform/methanol mixture (1:2) and left in a dark place overnight after adding $CaCl_2$. The chloroform layer was removed, evaporated first using an evaporator and then in an oven at 105°C for 30 min. The lipid content was calculated using differences between flask weights.

Fatty acid methyl esters (FAME) analyses

Fatty acid profiles from the seaweed samples were determined by gas chromatography (GC) of methyl esters. Boron trifluoride/methanol was used to prepare the fatty acid methyl esters (AOAC, 1990).

Gas chromatographic conditions

The fatty acid composition was analyzed using a GC Clarus 500 with autosampler (Perkin Elmer, Shelton, CT, USA) equipped with a flame ionization detector and a fused silica capillary SGE column (30 m X 0.32 mm ID X 0.25 μm BP20 0.25, SGE Analytical Science Pty Ltd, Victoria, Australia). The oven temperature was 140°C, for 5 min, and increased to 200° at 4°C/min intervals and then increased to 220° at 1°C/min. The injector and detector temperatures were set at 220° and 280°C, respectively. The sample size was 1 μL and the carrier gas was held at 16 psi. The split ratio used was 1:100. Fatty acids were identified by comparing the retention times of FAME in the samples with a standard 37 component FAME mixture. Two replicate GC analyses were performed and the results are expressed in GC area % as a mean value ± standard deviation.

Mineral analysis

The mineral content of seaweeds was determined by the dry ash method. The samples (~1 g) were placed in a prewashed crucible with nitric acid and burned at 550°C for 24 h until grey ash was obtained. First, 5 mL of nitric acid

(Merck 65%) were added to the resultant ash, and the mixture was stirred on a hot plate until dry. Then, 10 mL of the same acid were added continuously until a clear solution was obtained. The mixture was then filtered through an ash-free filter paper and made up to 25 mL with ultra pure water. ICP-AES (Varian Model-Liberty series II, Victoria, Australia) was used to determine the elements. The operational parameters for ICP AES are indicated below; each element was quantified by preparing a four point calibration curve for individual elements and the results are expressed as ppm. Pump Rate: 15 rpm; Plasma Gas: 15L/min; Fast Pump: On; PMT Voltage: 650 V; Auxiliary Gas flow: 1.5 L/min; Rinse Time: 10 sec.

RESULTS AND DISCUSSION

Proximate composition

The proximate composition of the seaweeds expressed on a dry weight basis is shown in Table 1. The protein content of macroalgae ranged from 7.2% for *J. rubens* to 35.40% for *P. pavonia*. The protein contents of marine algae depend on species and season (FLEURENCE, 1999; ABDEL-FATTAH and SARY, 1987). The protein content of the brown seaweeds was low (5-15% of the dry weight) compared to those of red and green seaweeds (10-30% of the dry weight) (BURTIN, 2003). In this study, the brown seaweeds *C. corniculata* and *P. pavonia,* had 30.43 and 35.40% protein on a dry weight basis, respectively. The red seaweeds, *L. papillosa* and *J. rubens* had protein contents of 34.65 and 7.2% dry weight, respectively.

The lipid level of seaweeds is generally low (1-5% of dry matter), and similar results were found in this study. The lipid content of seaweeds ranged from 6.73% for *L. papillosa* to 0.12% for *J. rubens* as dry weight. Although *J. rubens* had a significantly lower (0.12%) fat content ($P<0.05$), its ash content was notably higher (80.73%) ($P<0.05$) on a dry weight basis than the other seaweed species. The ash content of the seaweed species ranged from 28.97 to 80.73%. *J. rubens* had the highest ash content. The neutral detergent fibre level ranged from 14.01% for *C. corniculata* to 11.62% for *J. rubens*.

Fatty acid composition

Table 2 gives the percentage as a mean value of 20 fatty acids for each seaweed species. The fatty acid compositions of the seaweed species varied from 31.66 to 47.97% in saturated (SFA), 23.04 to 32.06% in monounsaturated (MUFAs) and 11.58 to 18.08% in polyunsaturated acids (PUFAs). Among them, palmitic acid (C16:0, 30.93-40.64%), myristoleic acid (C14:1, 2.52-6.02%), palmitoleic acid (C16:1, 2.98-18.84%), oleic acid (C18:1n9 cis, 1.37-23.17%), linoleic acid (C18:2n6, 0.95-4.25%), arachidonic acid (C20:4n6, 3.21-9.42%), and cis-5, 8,

Table 1 - The proximate composition of seaweeds expressed on dry weight basis (%).

Seaweed	Dry matter	Protein	Lipid	Ash	Neutral detergent fibre
Cystoseira corniculata	29.54	30.43±0.36	5.45±0.17	28.97±0.56	14.01±1.10
Padina pavonia	25.62	35.40±0.56	5.89±0.24	44.92±1.60	13.64±0.75
Laurencia papillosa	19.91	34.65±0.48	6.73±0.33	44.05±1.10	13.55±1.36
Jania rubens	65.45	7.20±0.19	0.12±0.01	80.73±3.16	11.62±1.40

Values are expressed as means ± standard deviation (n=3).

Table 2 - Fatty acid profiles of seaweed species.

Fatty acids (%)	*Cystoseira corniculata*	*Padina pavonia*	*Laurencia papillosa*	*Jania rubens*
C14:0	0.27±0.01	0.49±0.04	0.71±0.04	1.29±0.08
C15:0	0.35±0.02	0.49±0.02	0.72±0.06	1.36±0.47
C16:0	30.93±0.68	33.23±0.07	38.00±0.47	40.64±1.82
C18:0	0.11±0.01	0.31±0.01	0.49±0.01	0.61±0.06
C22:0	-	1.47±0.05	-	-
C23:0	-	-	1.40±0.01	4.07±0.04
∑ SFA	**31.66**	**35.99**	**41.32**	**47.97**
C14:1	4.73±0.11	6.02±0.00	4.42±0.10	2.52±0.21
C15:1	0.51±0.01	0.11±0.06	-	-
C16:1	2.98±0.23	12.49±0.47	17.75±4.34	18.84±0.67
C18:1n9	23.17±0.30	13.13±0.39	4.03±0.28	1.37±0.04
C20:1	0.25±0.03	0.26±0.01	-	0.31±0.01
C22:1n9	0.08±0.01	0.05±0.02	-	-
∑ MUFA	**31.72**	**32.06**	**26.02**	**23.04**
C18:2 n6	4.04±0.08	4.25±0.25	2.14±0.49	0.95±0.16
C18:3 n6	0.33±0.06	0.93±0.44	0.82±0.69	0.13±0.01
C18:3 n3	1.08±0.02	2.96±0.09	-	-
C18:4 n3	0.20±0.01	0.69±0.00	-	-
C20:2 cis	0.16±0.01	-	-	-
C20:4 n6	9.42±0.20	5.58±0.24	4.94±0.06	3.21±0.06
C20:5 n3	2.85±0.23	0.57±0.01	4.69±0.42	7.29±0.16
C22:6 n3	-	0.10±0.05	-	-
∑ PUFA	**18.08**	**15.08**	**12.59**	**11.58**
PUFA/SFA	**0.57**	**0.41**	**0.30**	**0.23**
∑n6	**13.79**	**10.76**	**7.9**	**4.29**
∑n3	**14.89**	**4.32**	**4.69**	**7.29**
n6/n3	**0.92**	**2.49**	**1.68**	**0.58**
Unidentified	**18.54**	**16.87**	**20.07**	**17.41**

Values are expressed as means ± standard deviation (n=2).

11, 14, 17-eicosapentaenoic acid (EPA, C20:5n3, 0.57-7.29%) had the highest proportions. These results are in agreement with previous studies on fatty acids in other species (BURTIN, 2003; KAMENARSKA *et al.*, 2002). Docosahexaenoic acid (DHA, C22:6n3) was only detected in *P. pavonia*.

In general there was a dominance of SFAs and MUFAs, whereas the proportion of PUFAs was low in all of the seaweed species. The highest proportion of PUFAs-n3 was found in *C. corniculata* (14.89%), followed by *J. rubens* (7.29%), *L. papillosa* (4.69%) and *P. pavonia* (4.32%). The highest PUFAs-*n*6 values were obtained from *C. corniculata* (13.79%), followed by *P. pavonia* (10.76%), *L. papillosa* (7.9%) and *J. rubens* (4.29%). The U.K. Department of Health recommends an ideal ratio of n6/n3 of 4.0 at the maximum (DEPARTMENT OF HEALTH, 1994). Values higher than the maximum value are harmful to human health and may promote cardiovascular diseases (MOREIRA *et al.*, 2001). In this study, the ratio of n6/n3 ranged from 0.58 for *J. rubens* to 2.49

for *P. pavonia*. The minimum PUFA/SFA ratio recommended was 0.45 (DEPARTMENT OF HEALTH, 1994). The highest PUFA/SFA ratio was obtained for *C. corniculata* (0.57) followed by *P. pavonia* (0.41), whereas the lowest values were found for *L. papillosa* (0.30) and *J. rubens* (0.23).

The main acids found in *C. corniculata* and *P. pavonia* (brown seaweed) were palmitic acid, followed by oleic acid, myristoleic acid, arachidonic acid and linoleic acid. Similar results were found by KAMENARSKA *et al.* (2002) who investigated the fatty acid content of *Padina pavonia* from the southern Adriatic Sea. Palmitic acid was the most abundant fatty acid in algae as in this study. The proportion of palmitoleic acid (C16:1) (12.49%) in *P. pavonia*, was higher than in *C. corniculata* (2.98%). Behenic acid (C22:0) and DHA (C22:6n3) were detected in *P. pavonia*, whereas eicosadienoic acid (C20:2cis) was only detected in *C. corniculata*. The EPA content was also higher (2.85%) in *C. corniculata* than in *P. pavonia* (0.57%).

Palmitic acid, palmitoleic acid and EPA were the major fatty acids found in red seaweeds (*L. papillosa* and *J. rubens*). Eicosanoic acid (C20:1) was only detected in *J. rubens*, which had a higher EPA content (7.29%) than *L. papillosa* (4.69%). The red seaweed species investigated in this study had higher levels of palmitic acid, palmitoleic acid and EPA than the brown seaweeds, whereas brown seaweed had higher proportions of oleic and arachidonic acids. Behenic acid (C22:0), cis-10-pentadecanoic acid (C15:1), linolenic acid (C18:3n3), octadecatetraenoic acid (C18:4n3) and cis-11, 14-eicosadienoic acid (C20:2) were not detected in the red seaweed species. The seaweeds had high levels of SFA and MUFAs and also contained a considerable amount of PUFAs. These findings indicate that these seaweeds could be used as a source of PUFAs in the formulation of diets.

Mineral content

Since the metabolic system of seaweeds can absorb elements directly from the seawater, they are rich minerals (NORZIAH and CHING, 2000). It is therefore, important to determine the mineral content in seaweed for nutritional and toxicological purposes (SUBBA RAO *et al.*, 2007). The macro- and micro-minerals found in the seaweeds are presented in Table 3. The Ca content was higher in red algae (7,953.03 μg/g d wt for *L. papillosa* and 8,837.78 μg/g d wt for *J. rubens*) compared to the brown algae (1,161.62 μg/g d wt for *C. corniculata* and 2,256.47 μg/g d wt for *P. pavonia*). The Mg content in the red seaweeds was almost twice that found in brown seaweeds. The highest Na and K levels were found in *L. papillosa* (252.85 μg/g d wt and 863.30 μg/g d wt, respectively), whereas *C. corniculata* had the highest amount of P (45.30 μg/g d wt, followed by *L. papillosa* (14.51 μg/g d wt). The mineral contents of seaweeds were generally lower than those reported by RUPEREZ (2002) for brown seaweeds (*Fucus vesiculosus, Laminaria digitata, Undaria pinnatifida*) and red seaweeds (*Chondrus crispus* and *Porphyra tenera*). Macro mineral contents when expressed as Na+K+Ca+Mg in land vegetables such as potato (6,015), sweet corn (1,347) and carrots (3,276) (USDA, 2001) are all lower than those of seaweeds, except spinach (9,679), which has a value comparable to that of red algae (*L. papillosa* and *J. rubens*). Na/K ratios in all the seaweeds studied ranged from 0.20 to 1.23. High Na/K ratios are related to hypertension (RUPEREZ, 2002).

The levels of trace elements found in the seaweeds are given in Table 3. The lowest Zn content was found in *L. papillosa* (2.56 μg/g d wt), while the highest level was found in *J. rubens* (3.99 μg/g d wt). The Mn content ranged from 0.78 to 4.93 μg/g d wt and was higher in red algae than in brown seaweeds (Table 3). The mineral content varies according to

Table 3 - Mineral content of seaweed species (μg/g dry weight).

Macro elements	*Cystoseira corniculata*	*Padina pavonia*	*Laurencia papillosa*	*Jania rubens*
Ca	1,161.62±4.41	2,256.47±6.32	7,953.03±92.26	8,837.78±76
Mg	271.08±1.68	218.60±1.40	446.17±2.41	573.32±0.69
Na	26.55±0.29	48.13±0.50	252.85±2.15	60.63±0.04
P	45.30±0.25	11.31±0.04	14.51±0.07	6.86±0.03
K	131.53±0.88	129.05±0.49	863.30±8.72	49.21±0.20
Na+K+Ca+Mg	1,590.7	2,652.29	9,515.35	9,520.94
Na/K	0.20	0.37	0.29	1.23
Trace elements				
Zn	3.65±0.02	3.05±0.02	2.56±0.03	3.99±0.02
Mn	0.78±0.0	2.03±0.02	4.93±0.03	3.85±0.01

Values are expressed as means ± standard deviation (n=3).

species, locality, season, environmental factors and processing methods (YOSHIE *et al.*, 1994; HONYA *et al.,* 1993).

In conclusion, this study has shown that seaweeds (*J. rubens*, *L. papillosa, C. corniculata, P. pavonia*) have a high nutritional value in terms of protein, fatty acids (especially PUFAs), and macro and trace mineral elements. These seaweeds could be used as a food supplement to improve the nutritive value of human or animal diets, especially as a source of minerals due to their rich macro mineral and trace element content.

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SHORT COMMUNICATION

EFFECTS OF OZONE AND NEGATIVE AIR IONS (NAI) ON THE *IN VITRO* DEVELOPMENT OF *BOTRYTIS CINEREA* AND *PENICILLUM EXPANSUM* MOULDS

EFFETTI DELL'OZONO E DELL'ARIA IONIZZATA SULLO SVILUPPO *IN VITRO* DI *BOTRYTIS CINEREA* E *PENICILLUM EXPANSUM*

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ABSTRACT

The effects of Ozone and negative air ions (NAI) on the growth rate of *Botrytis cinerea* and *Penicillium expansum* colonies, and on sporulation, have been investigated. Inoculated plates were stored in Air (control), or air with Ozone (0.5 ppm), NAI ($6.1x10^6$ ioni/cm^3) and a NAI+Ozone ($6.1x10^6$ ioni/cm^3 + 0.5 ppm) mixture at 7°C and 95% RH. After 48 h a reduced growth rate of the

RIASSUNTO

È stata studiata l'efficacia dell'ozono e/o dell'aria ionizzata (NAI) sulla velocità di crescita e sulla produzione di spore di colonie di *Botrytis cinerea* e *Penicillum expansum*. Le muffe sono state inoculate su piastre di potato dexstrose agar (PDA) e sottoposte a trattamento con Aria (controllo), miscela Aria-Ozono (0,5 ppm), miscela Aria-NAI ($6,1x10^6$ ioni/cm^3), miscela Aria-NAI+Ozono ($6,1x10^6$

- Key words: *Botrytis cinerea*, fungistats, NAI, Ozone, *Penicillum expansum*, post-harvest -

cultures stored in the presence of the fungistatic agents was observed. The reduction was greater when *B cinerea* and *P. expansum* were exposed to NAI+Ozone (30 and 83%, respectively). Ozone stopped the production of *P. expansum* spores and delayed spore production of *B. cinerea*.

ioni/cm^3 e 0,5 ppm) a 7°C e circa 95% UR. Durante i primi due giorni è stata osservata una riduzione della velocità di crescita delle colture sottoposte agli agenti fungistatici; in seguito la velocità di crescita fungina aumenta fino a raggiungere condizioni normali e costanti. La riduzione è stata maggiore quando le muffe sono state esposte a ozono e ioni negativi contemporaneamente. *P. expansum* sembra essere più sensibile all'azione degli agenti fungistatici rispetto a *B. cinerea*. È stato osservato anche che l'ozono ha inibito reversibilmente la produzione di spore di *P. expansum* e ritardato quella di *B. cinerea*.

INTRODUCTION

In recent years, the social and economic importance of food quality and safety issues has increased. Consumer awareness of produce quality and pesticide use requires the industry to consistently improve quality while aiming to minimize pesticide use on produce. In the food industry, the sanitization of equipment and the treatment of food is currently carried out by chemical or physical means. Thermal treatment, especially steam, is effective in destroying contaminating microorganisms but is quite expensive to generate and is not applicable if high temperature negatively affects food quality or equipment integrity (TROLLER, 1993). Irradiation is not recommended in food processing plants because of the inherent hazards for the operators associated with a radioactive source. Chemicals, such as derivatives of chlorine, acids, bases, hydrogen peroxide, iodine, and quaternary ammonium compounds are currently the most common by used sanitation methods in the food industry. Each has some significant disadvantages, e.g. iodine has a pungent smell, is expensive, corrosive and may cause irritation; hydrogen peroxide is less effective, tends to degrade and loses its sanitizing activity if not properly handled; quaternary ammonium compounds, while odourless, colourless and flavourless, are chemically very stable and therefore they can remain on food for a long time. Chlorine and its derivatives are used worldwide for water and wastewater treatment, food washing and the cleaning-in-place (CIP) procedure for processing plants and equipment. These compounds are not expensive, are effective against all kinds of vegetative cells and spores, and play an important role in the control of food-borne diseases, as well as in the prevention of fruit and vegetable spoilage. However, chlorinated agents also have some serious disadvantages; they require large amounts of water for rinsing, are harmful and are irritant in humans at high concentrations and are not environmentally friendly (BELLAR *et al.*, 1974). Moreover, chlorine reacts with many organic compounds, e.g., aquatic humus

compounds, to form by-products, such as trihalomethanes and haloacetic acids, that have been judged mutagenic and carcinogenic (TRUSSELL and UMPHRES, 1978; GOPAL *et al.*, 2007). These substances can contaminate food and drinking water, adversely affecting public health and the native aquatic and terrestrial species. Despite the hazards of forming carcinogenic and mutagenic compounds, chlorine and its derivates are still widely used because the lack of adequate sanitizers would result in a worse situation such as much more illness and loss of life due to microbial contamination of food and water (GUZELSEYDIM *et al.*, 2004).

The food industry is currently looking for innovative technologies that are environmentally sound and meet consumer demand for fresh, safe ready-to-eat products. In addition, new sanitizing agents should be non-corrosive for expensive processing equipment, and should not affect foodstuff quality. Ozone seems to meet all of these needs; it is one of the most potent sanitizers due to its high oxidation potential (E° = +2,076 V) (MANLEY and NIEGOWSKI, 1967). It is active against all forms of microorganisms at relatively low concentrations, and an excess of Ozone rapidly auto-decomposes into oxygen, leaving no chemical residues on food (KARACA and VELIOGLU, 2007).

Gas-phase Ozone treatment of fruits and vegetables has been used to increase shelf-life (NORTON *et al.*, 1968). Ozone has also been used to prevent surface contamination on grapes and blackberries (SARIG *et al.*, 1996; BEUCHAT, 1992), whole and sliced tomatoes (AGUAYO *et al.*, 2006), broccoli florets (ZHUANG *et al.*, 1996), and whole black peppercorns and ground black pepper as a substitute for ethylene oxide (ZHAO and CRANSTON, 1995). The increased shelf-life of apples, oranges, tomatoes, stone fruits, melons and kiwi fruits after Ozone treatment has been attributed to the oxidation of ethylene (RICE *et al.*, 1982; BARRY and GIOVANNONI, 2007; LOVINO *et al.*, 2007). Low concentrations (<1 ppm) and prolonged contact time (several days) are required to inhibit microbial growth during storage of kiwi fruit (LOVINO *et al.*, 2006).

The sanitizing mechanism of Ozone consists mainly in the deactivation of intracellular enzymes, probably due to the oxidation of sulfhydryl groups into cysteine residues (CHANG, 1971), the oxidation of various components of the cell envelope, including polyunsaturated fatty acids, membrane-bound enzymes, glycoproteins and glycolipids, that cause leakage of the cell contents and cell lysis (SCOTT and LESHER, 1963; MURRAY *et al.*, 1965), and the reaction with nucleic acids, especially thymine, (SCOTT, 1975; ISHIZAKI *et al.*, 1981), and proteins of bacterial spore coats (FOEGEDING, 1985) and of viral capsides (SPROUL and KIM, 1980; KIM *et al.*, 1980). High Ozone concentration are required to rapidly kill fungi or pathogen spores that cause citrus spoilage, i.e., blue mould, sour rot and green mould (SMILANICK, 2003). However, excessive concentrations of Ozone may cause oxidation of food surfaces (especially on Ozone-sensitive products) resulting in discouloration and the deterioration of flavour and aesthetic characteristics (RICE *et al.*, 1982). In addition, high Ozone concentrations require corrosion-resistant facilities and appropriate safety measures. Currently, Ozone is ordinarily used in drinkable-water treatment plants in Europe. In the Usa, the Food and Drug Administration has decreed Ozone to be a Generally Recognized as Safe (GRAS) substance and has approved its use in gas and aqueous phases as an additive for the treatment, storage, and processing of food (FEDERAL REGISTER, 2001).

There is a debate about the sanitizing effects of negative air ions (NAI) on living organisms but researchers agree that they can be bacteriostatic (KRUEGER and REED, 1976; CHARRY and KAVET, 1987; REITER, 1993; TANIMURA *et al.*, 1997).

NAI has been shown to reduce airborne microbial contamination in dental clinics (GABBAY *et al.*, 1990), *Salmonella* contamination in chicken hatcheries (GAST *et al.*, 1998), and the airborne spread of viruses in poultry houses (MITCHELL and KING, 1994). Studies by TANIMURA *et al.* (1998, 1999) and FAN *et al.* (2002) showed that the sanitizing activity of Ozone against some bacteria was improved when used in combination with NAI; no explanation of the mechanism of this synergism was given.

This synergism could improve the efficacy and lower the doses of Ozone. The goal of this study was to compare the fungistatic action of both Ozone and NAI on two important spoilage moulds, *Botrytis cinerea* and *Penicillum expansum*, and to determine if there is a synergic effect of the NAI+Ozone mixture.

MATERIALS AND METHODS

Pure cultures of *Botrytis cinerea* Pers. and *Penicillum expansum* Link were treated with cold forced air or with cold forced Ozone, NAI and NAI+Ozone in a pilot plant. The growth inhibition was determined by measuring the colony growth rates and the inhibition of sporulation; its reversibility was determined by incubating the treated fungi at 3°, 5° and 25°C.

Pilot Plant

The stainless steel pilot plant (prototype from Ionex s.r.l., Vicenza, Italy), shown in Fig. 1, had a volume of 1 m^3 and consisted of a treatment chamber and a fan that generated airflow. The input air was taken from the ambient air and was first enriched with Ozone produced by a corona discharge generator (-8 kV Ozone generator, Ionex s.r.l., Vicenza, Italy), and then with NAI also produced by a corona discharge generator (-12 kV NAI generator, Ionex s.r.l., Vicenza, Italy). The gas mixture was recycled in the test chamber by a fan. The output air was filtered through an activated charcoal filter. The Ozone and NAI concentrations in the test chamber were set by regulating the inlet and outlet valves, in other words by regulating the quantity of Ozone free - fresh air introduced into the pilot plant. The pilot plant was installed in a 20 m^3 indus-

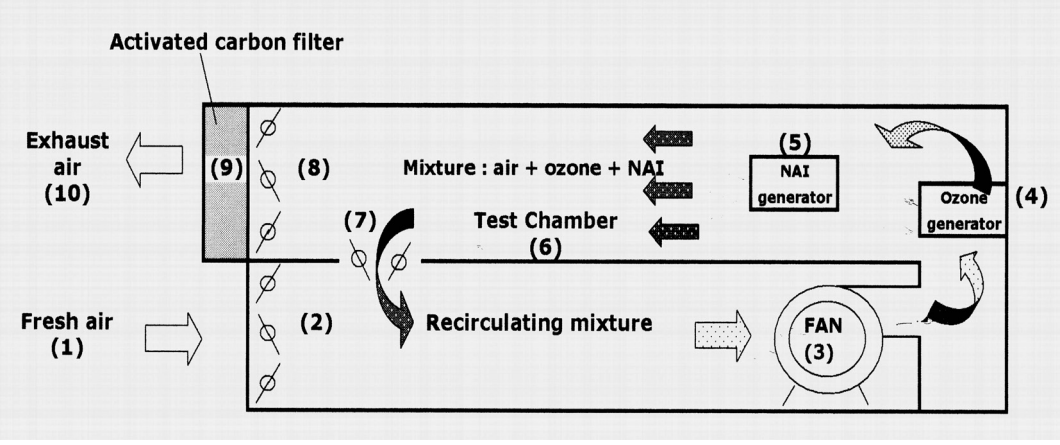


Fig. 1 - Pilot plant scheme. Legend: (1) inlet air; (2) inlet air gate; (3) ventilation fan; (4) -8 kV corona discharge Ozone generator; (5) -12 kV corona discharge NAI generator; (6) test chamber; (7) recirculation air gate; (8) outlet air gate; (9) activated carbon filter; (10) outlet air.

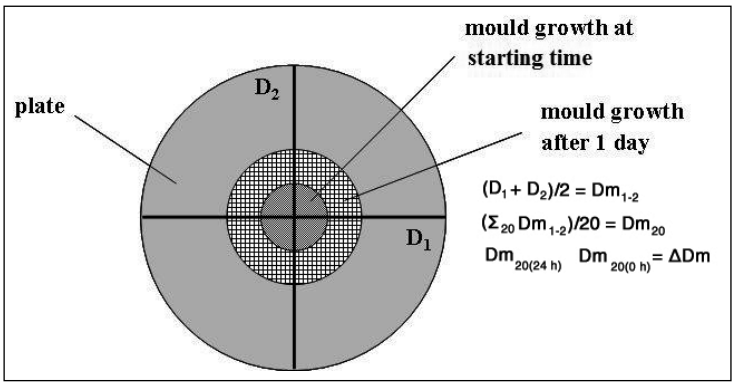


Fig. 2 - Methodology used to calculate variation of mean diameter of fungal colonies in the time interval 0-24 h; Dm_{1-2} is the mean between the two perpendicular diameters of each plate; $Dm_{20(0\ h)}$ and $Dm_{20(24\ h)}$ are the mean values of twenty plates calculated at 0 hour and after 24 hours, respectively.

trial cold storage room that was maintained at 7±1°C and high relative humidity (95-98% RH) operating with partial recirculation of air. Inside the treatment chamber, a 0.7 m/s air flow was maintained and the Ozone and NAI concentrations were set at 0.5±.05 ppm (v/v) and about $6.1x10^6$ ions/cm^3, respectively. The Ozone concentration was monitored continuously by an Ozone analyser (model OS-4, Eco Sensors, Inc, Santa Fe, New Mexico, Usa), while the NAI concentration was measured with an ion detector (Air Ion Counter, Alpha Lab, Inc., Salt Lake City, UT, Usa). A thermo-hygrometer (model 635, Testo, Milan, Italy) was used to check temperature and air humidity in both the pilot plant and in the cold storage room.

Mycelia growth rate experiments

Pure cultures of *B. cinerea* and *P. expansum* were maintained on potato dextrose agar (PDA) in test tubes at 4°C. At the start of each test, young cultures were prepared by incubating the moulds on PDA medium at 25°C for 10 days (5 days under lamp light and 5 days in the dark). Agar plugs of about 5 mm diameter were taken from the margins of 15-day-old cultures and aseptically placed in the centre of 20 PDA Petri plates (NADAS *et al.*, 2003). After one day of incubation at 25°C, the border of each colony was marked to determine the colony size at the starting time. In addition, two perpendicular lines were traced across the centre of the colony. Colony growth rate was evaluated by measuring the diameters on the two perpendicular lines. Subsequently, the twenty plates, with their lids removed, were placed randomly inside the treatment chamber (in 5 lines of 4 plates each), and continuously exposed to one of the following treatments: Air, Ozone, NAI or NAI+Ozone. The treatment time for tests was fixed at 96 h (120 h for Air and Ozone on *B. cinerea*). Every 24 h, two orthogonal diameters of the colonies of each kind of mould were measured with calipers and a stereoscope. For each exposure time, the mean diameter of the colony was calculated as the average of twenty values. Fig. 2 shows the methodology used to calculate the variation of mean diameter (ΔDm), for example, over the time interval 0-24 h.

The experiments with *B. cinerea* and *P. expansum* were carried out separately. Each treatment was repeated at least twice. The data were subjected to an analysis of variance (ANOVA) and the means were separated by Duncan's multiple range test (with $P<0.05$) to determine if there were significant differences in the radial growth rate of moulds,

with respect to the mean value of the experiments.

It was observed that the position of the plates inside the pilot plant did not influence the radial growth rate and effectiveness of each treatment.

Sporulation control experiments

The same *P. expansum* plates used for the growth rate test were monitored daily to determine mould sporulation under different environmental conditions (Air, Ozone, NAI, or NAI+Ozone). Other incubated control plates were placed in an incubator at 25°C for one day, and then in other rooms at different temperatures and 95-98% RH. The presence of green-coloured spores (indicating sporulation) was determined by examining the cultures under a stereomicroscope.

To determine if the inhibition of mould sporulation was reversible, plates from the NAI+Ozone test were subsequently placed in rooms at different temperatures: Cold Room 1, Cold Room 2 and Incubator. Table 1 gives the details of the treatment conditions.

Each test lasted up to 120 h. The plates used for the NAI+Ozone experiment were tested for an additional 96 h to determine the reversibility of sporulation inhibition. The following qualitative scale was used to characterize sporulation: - spores absent; + start of sporulation; ++ spores present (PALOU *et al.*, 2002).

It was not possible to carry out this test with *B. cinerea* because its sporulation time is much longer than that of *P. expansum*. Consequently, the extended exposure of the open Petri plates to the airflow caused the PDA to dehydrate making the test impossible. However, as an alternative test, after 96 h in the NAI+Ozone the *B. cinerea* treatment plates were placed in a cold storage room at 3°C and 95-98% RH. The results were compared with *B. cinerea* colonies grown from the beginning under control conditions (3°C and 95-98% RH) and with colonies grown at 25°C.

Table 1 - Operating conditions in the treatment chambers.

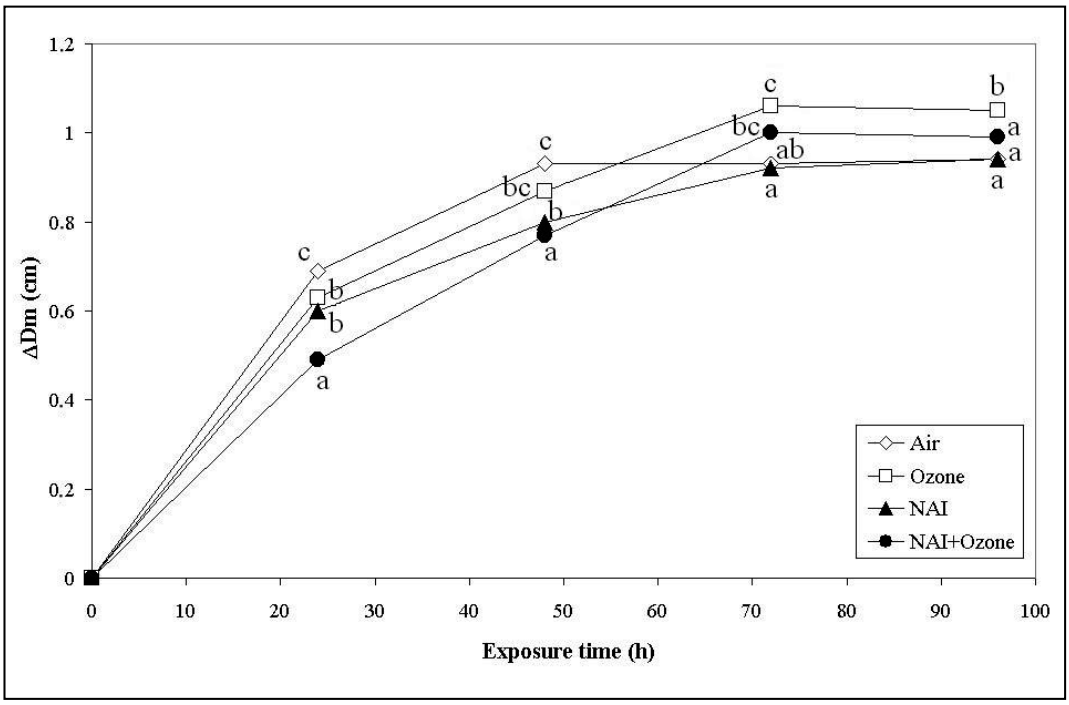
Treatment	T (°C)	[Ozone] (ppm)	[NAI] (Ions/mL)
Pilot Plant - Air	7	-	-
Pilot plant - Ozone	7	0.5	-
Pilot Plant - NAI	7	-	6.1×10^6
Pilot plant - NAI+Ozone	7	0.5	6.1×10^6
Cold Room 1 - Air	3	-	-
Cold Room 2 - Air	5	-	-
Incubator - Air	25	-	-

RESULTS AND DISCUSSION

In vitro mycelia growth

The radial growth rate of the fungal cultures, subjected to the different treatments, is reported in Fig. 3. The growth rate is expressed as a variation of the mean diameter of the fungal colony as a function of time. The radial growth of *P. expansum* was significantly reduced by all treatments compared to Air during the first 48 h; *B. cinerea* growth was significantly reduced by all treatments during the first 24 h, but was reduced only by NAI and NAI+Ozone treatments at the end of 48 h. After 48 h of any treatment on *P. expansum* and of the NAI and NAI+Ozone treatments on *B. cinerea*, it was observed that the colony growth rate was not statistically different from that in Air. With the Ozone treatment on *B. cinerea*, the colony growth rate was statistically different from that in Air at 72 and 96 h, but the results were not statistically different from those with Air at 120 h (data not reported).

The fungal colonies were able to overcome the inhibitory effect of the fungistats and their development was similar to the control colonies without NAI and/



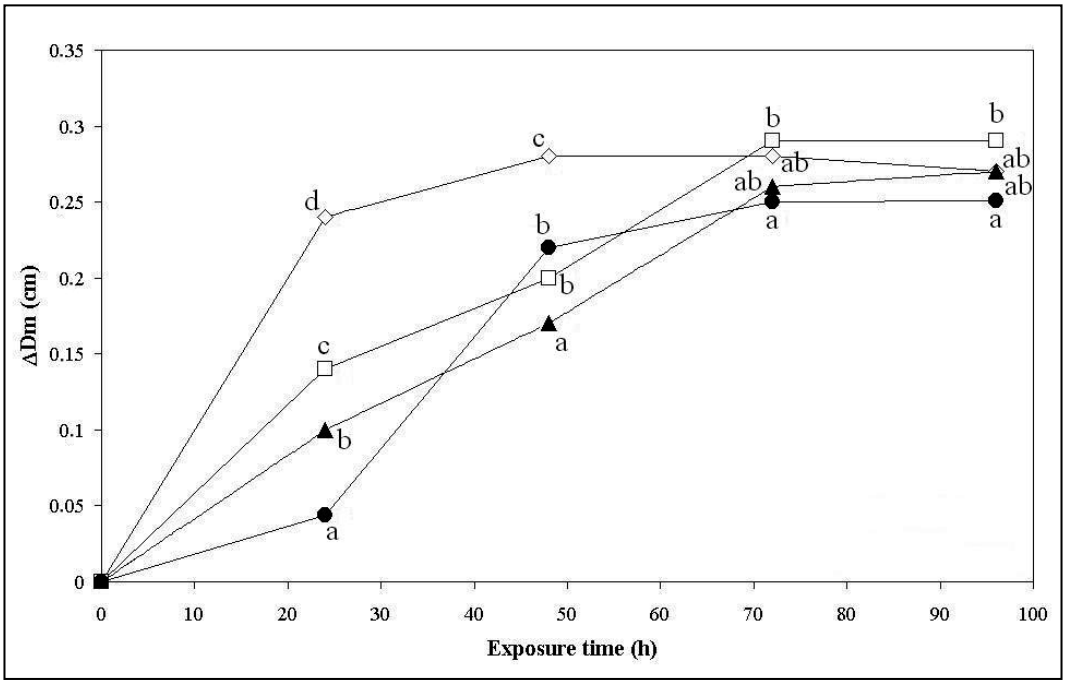


Fig. 3 - Effect of Ozone (0.5 ppm) and/or negative air ions ($6.1x10^6$ NAI/mL) on radial growth rate of *B. cinerea* (a) and *P. expansum* (b). Different letters on value indicate a statistically significant difference ($P \leq 0.05$) according to the Duncan Test.

or Ozone. This fact can be explained by assuming that the fungistatic action occurred during the initial growth period (24-48 h), while the moulds adjusted to the new environmental conditions and probably when they were more vulnerable to the hostile agents.

The best results were achieved with NAI+Ozone treatment at 24 h; the *B. cinerea* and *P. expansum* colony diameters were reduced 30 and 83%, respectively; the effect was synergistic on *B. cinerea* (e.g., Ozone 9% and NAI 13% at 24 h) in the first 48 h.

P. expansum was more sensitive to fungistats than *B. cinerea*; this could be due to the growth limit temperature of *P. expansum* (0°C) compared to that of *B. cinerea* (- 4°C) (RAPER and THOM, 1949; ELAD and EVENSEN, 1995). At the first sight, the effectiveness of the NAI+Ozone treatment, like the others, could appear to be limited. However, it should be considered that all of the treatments were carried out on already developed fungal colonies on PDA, a medium that provides nutrients and physical protection for the hyphae. Better results would be expected under real conditions of fruit and vegetable treatment, because their surfaces do not provide the same favourable growing conditions, as observed by PALOU *et al.* (2001), who found that Ozone is especially effective on the non-protected aerial growth of mycelia.

NADAS *et al.* (2003) observed a 90 and 40% reduction in the *B. cinerea* colony growth, inoculated on PDA plates at 2°C, 90% RH and in an Ozone-enriched atmosphere (1.5 ppm), after 12 and 18 days, respectively. Similar results were obtained by LIEW and PRANGE (1994) regarding the daily growth rate of *B. cinerea* exposed to 60 ppm Ozone at 2°, 8° or 16°C. In contrast, PALOU *et al.* (2001) observed little or no difference in the radial growth of *P. italicum* and *P. digitatum* colonies exposed to 0.3 ppm Ozone at 5°C and 90% RH. KLOTZ (1936) reported similar observations on the same microorganism. Therefore it seems that the inhibition of fungal growth is improved by increasing the fungistat concentration.

Sporulation experiments

The results on sporulation tests of *P. expansum* are reported in Table 2.

The sporulation of the control started after 24 h with the Air treatment. In the case of both the Ozone and NAI+Ozone treatments, sporulation was suppressed

Table 2 - Influence of continuous 0.5 ppm Ozone and/or 6.1×10^6 NAI/mL exposure on sporulation of *Penicillum expansum*. Sporulation rating: - spores absent; + start of sporulation; ++ spores present. The percentages refer to the number of colonies at a certain sporulation rating.

Treatment	Exposure time (h)					
	0	**24**	**48**	**72**	**96**	**120**
Pilot plant - Air	100% -	90% + 10% -	90% ++ 10% +	100% ++		
Pilot plant - Ozone	100% -	100% -	100% -	100% -	100% -	
Pilot plant - NAI	100% -	100% -	100% -	90% + 10% ++	90% + 10% ++	
Pilot plant - NAI+Ozone	100% -	100% -	100% -	100% -		
Cold Room 1 (3°C)	100% -	100% -	67% + 33% -	67% + 33% -	67%++ 33% +	100% ++
Cold Room 2 (5°C)	100% -	100% +	100% ++			
Incubator (25°C)	100% -	100% ++				

Table 3 - Influence of continuous 0.5 ppm Ozone and $6.1x10^6$ NAI/mL exposure on sporulation of *Penicillum expansum* after treatment. Sporulation rating: - spores absent; + start of sporulation; ++ spores present. The percentages refer to the number of colonies at a certain sporulation rating.

Treatment	Exposure time (h)				
	120	**144**	**168**	**192**	**216**
Cold Room 1 (3°C)	100% -	100% -	100% +	100% ++	
Cold Room 2 (5°C)	100% -	100% -	50% -	50% +	100% ++
			50% +	50% ++	
Incubator (25°C)	67% -	100% ++			
	33% +				

during the 96 h experiment, while NAI treatment only delayed sporulation; formation of spores was detected after 72 h. Therefore it is reasonable to suppose that the inhibition of sporulation is due to Ozone rather than NAI. For the other three treatments, temperature was a decisive factor: at higher temperatures spore formation occurs more rapidly.

The plates exposed to the NAI+Ozone treatment were divided into three groups and were kept in an incubator and in two different cold rooms for varying lengths of time (Table 3).

After the elapsed times that varied with the treatment, all of the fungal colonies resumed normal sporulation. These results demonstrate that the inhibition of sporulation by Ozone is reversible once the Ozone is removed. Several other authors (PALOU *et al.*, 2001; HARDING, 1968) have shown that the sporulation of other *Penicillium* species was inhibited only as long as Ozone was present.

The delay in *B. cinerea* sporulation was longer than that of *P. expansum* after exposure to NAI+Ozone because it has a longer life cycle than *Penicillium*. The fungal colonies grown at 25° or 3°C produced conidia after 5 and 13 days, respectively. In contrast, the moulds stored first in the pilot plant under NAI+Ozone treatment for 96 h and then in a cold store room at 3°C, started sporulation after 20 days. Similar findings have been reported by several Authors (TZORTZAKIS *et al.*, 2008; PALOU *et al.*, 2002; SPALDING, 1966, 1968; RIDLEY and SIMS, 1967), but only for Ozone treatment and at different concentrations. HIBBEN and STOTZKY (1969) concluded that spore sensitivity to the oxidizing action of Ozone depended on the fungal species, spore morphology, substrate, moisture, and Ozone dosage. As previously reported, in an *in vitro* test, while Ozone gas and NAI do not kill spores or affect germination, they do inhibit normal mycelial growth (at least initially) and greatly reduce sporulation (especially Ozone gas). The inhibition of fungal sporulation by Ozone and NAI treatment on infected fruits and vegetables in storage could prevent the spread of fungal contamination from infected fruit and vegetables to adjacent healthy ones as well as to packages, room walls and floors. Storage under Ozone and NAI could greatly reduce the load of airborne pathogenic spores which would subsequently reduce spoilage in the stored produce. With the use of sanitizing treatments, the lack of sporulation on decayed fruits could reduce the use of fungicidal treatments and prevent the proliferation of fungicide-resistant strains of the pathogens. Usually, fruits stored at low temperature are treated with fungicides and, therefore, many spores produced during storage come from resistant strains. Any practice that reduces inoculum production on

decayed fruit after fungicide treatment, could help prevent and control contamination and resistance in target pathogen populations (PALOU *et al.*, 2001).

Overall, the best results were obtained with the combined NAI+Ozone treatment, probably due to a synergistic effect between the Ozone and NAI. FAN *et al.* (2002) reported a strong synergy between Ozone and NAI on bacterial cell death; the degree of this effect varied depending on the bacterial species. TANIMURA *et al.* (1998) obtained the best results; in *in vitro* experiments 0.05 ppm Ozone and $3.0x10^5$ NAI/cm^3 completely inhibited the germination and sporulation of *Aspergillus niger* mould. Several authors (LI *et al.*, 1989; HILDEBRAND *et al.*, 2001; SONG *et al.*, 2000) have found that different concentrations of Ozone gas and NAI reduced decay in mandarin oranges, table grapes and onions.

The probable interaction of Ozone and NAI could be exploited to control decay in cold stored fruits and vegetables. However, additional research is needed to understand how the nature and composition of their surfaces, type of microbial contaminants, fungistats concentration and environmental conditions affect the inactivation of microflora without harming the foodstuff, equipment and worker's health.

ACKOWLEDGEMENTS

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SHORT COMMUNICATION

ANTIOXIDANT ACTIVITY OF *PRUNUS SPINOSA* L. FRUIT JUICE

ATTIVITÀ ANTIOSSIDANTE DI SUCCO DI FRUTTI DI *PRUNUS SPINOSA* L.

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ABSTRACT

The purpose of this work was to assess the antioxidant activity of fresh juice from *Prunus spinosa* L. fruit (Rosaceae) growing wild in Urbino (central Italy) by using different *in vitro* analytical methods: 5-lipoxygenase test, 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging and ORAC (Oxygen Radical Absorbance Capacity). BHT, Trolox, and ascorbic acid were used as reference antioxidant compounds. In the 5-lipoxygenase test the fresh fruit juice of *P. spinosa* showed

RIASSUNTO

Lo scopo di questo lavoro è stato quello di valutare l'attività antiossidante del succo fresco di frutti di *Prunus spinosa* L. (Rosaceae), pianta spontanea delle campagne di Urbino (Italia centrale). Tale attività è stata studiata *in vitro* mediante tre test: quello della 5-lipoxygenase, del 1,1-diphenil-2-picrylhydrazyl (DPPH) free radical scavenging e ORAC (Oxygen Radical Absorbance Capacity). Come antiossidanti di riferimento sono stati considerati BHT, Trolox ed Acido ascorbico. Nel test

- Key words: anthocyanins, antioxidant activity, *Prunus spinosa* L. -

a good antioxidant activity when compared to Trolox; in the DPPH test the samples showed IC50 values that were comparable to ascorbic acid, while the ORAC value was 36.02 μmol eq. Trolox/g of fruit. These values are in accord with data reported in the literature for small fruits such as vaccinium, rubus and ribes. The antioxidant activity of red berries is correlated with the anthocyanin content. The results of this study indicate that the three most representative anthocyanins in sloe fruit juice (cyanidin-3-rutinoside, peonidin-3-rutinoside and cyanidin-3-glucoside) contribute in varying amounts to the antioxidant properties.

della 5-lipoxygenase, tale succo di frutta ha mostrato una buona attività antiossidante paragonabile al Trolox, nel test del DPPH il campione ha mostrato una IC50 comparabile a quella dell'Acido ascorbico, mentre il valore di ORAC pari a 36.02 μmol eq. Trolox/g di frutti è risultato in accordo con i dati riportati in letteratura per piccoli frutti tipo vaccinium, rubus e ribes. È noto che l'attività antiossidante dei frutti rossi è correlata al contenuto in antocianine: i nostri risultati mostrano che le tre principali antocianine presenti nel succo di frutti di prugnolo (cianidin-3-rutinoside, peonidin-3-rutinoside e cianidin-3-glucoside) contribuiscono all'attività antiossidante in percentuali differenti nei tre test utilizzati.

INTRODUCTION

Prunus spinosa L. (Rosaceae) known as "blackthorn" or "sloe" is a wild shrub, native to Scotland and commonly found in deciduous forests in Europe and temperate Countries in Asia, especially in central, north, west and south Anatolia (KUMARASAMY *et al.*, 2003). It is resistant to cold, drought, and calcareous soils and is one of the ancestors of *P. domestica* (APARAJITA *et al.*, 2002). The fruit is bluish black, bloomy, 5-7 mm globular drupes, with green astringent flesh; they are popularly called "sloes" and despite their succulent appearance are far too bitter for human consumption, except as flavouring in liqueurs and wine. In the Marche region (central Italy), *P. spinosa* fruit is used to make a wine called "Lacrima di Spino Nero" (Boll. Uff. Reg: Marche, 2002).

The medicinal properties of blackthorn fruit extracts (purgative, diuretic, disintoxicant) make them suitable for the preparation of natural medicines (APARAJITA *et al.*, 2002). Anthocyanins were detected in "sloe" fruits by WERNER *et al.* (1989), RAMOS and MACHEIX (1990), CASADO-REDIN *et al.* (1992) and DEINEKA *et al.* (2005), and cyanidin-3-glucoside and cyanidin-3-rutinoside with peonidin-3-glucoside and peonidin-3-rutinoside were identified as the main anthocyanins. Caffeoil-3'-quinic acid was the most abundant hydroxycinamic derivative and different quercetin glycosides were also detected.

Anthocyanins are widely distributed in fruit and vegetables. They are one of the main classes of water-soluble flavonoids and contribute significantly to the antioxidant activities of the flavonoids (LAPIDOT *et al.*, 1999). They are well known for their ability to give red, blue, and purple colours to plants. They are also free radical-scavengers and can potentially interact with biological systems, to confer enzyme-inhibiting, antibacterial, cardiovascular protection and

antioxidant effects (DE GROOT and RAUEN, 1998; COWAN, 1999). WANG and LIN (2000) found a strong correlation between antioxidant capacity, total phenols and anthocyanins, while other investigations have indicated that anthocyanins may be less significantly correlated with the antioxidant properties (FRANKEL *et al.,* 1995; BURNS *et al.*, 2000; AMOU *et al.*, 2002; KALLITHRAKA *et al.*, 2005). To date only seed extracts (KUMARASAMY *et al.*, 2004) and aqueous extracts (thea) from dried fruit of *P. spinosa* (KISELOVA *et al.,* 2006) have been tested for antioxidant activity.

In this paper, for the first time, the antioxidant activity of fresh *P. spinosa* juice is reported and compared to the activity of grape juice and to the activity of the three most representative anthocyanins (commercially available) contained in "sloe" juice.

MATERIALS AND METHODS

Chemicals

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), BHT (butylated hydroxytoluene), ascorbic acid, quercetin, 5-lipoxygenase, 2,2[I]-azobis(2-amidinopropane) dihydrochloride (AAPH), and fluorescein sodium salt were purchased from SIGMA (Milano, Italy).

Cyanidin-3-O-glucoside chloride, cyanidin-3-O-rutinoside chloride and peonidin.-3-O-rutinoside chloride were purchased from Extrasynthese (Lyon, France).

All other reagents and chemicals were of analytical grade and purchased from SIGMA.

Plant materials and juice preparation

P. spinosa fruit was collected in Urbino, the Marches, central Italy, at 500 m a.s.l., in November 2007 and identified by D. Fraternale. A voucher specimen is deposited in the herbarium of the Botanical Garden of the University of Urbino (P.S.F. 163). Juice was prepared as follows: 10 g of "sloe" fruit were crushed in a mortar and the mush was filtered through Whatman No. 1 filter paper under vacuum and the residue was squeezed until exhaustion. The juice, 6 mL, was then centrifuged at 2,500 rpm for 10 min and stored in ice.

Vitis vinifera L. fruit (Sangiovese cultivar, the Marches, Italy) was collected from the vineyards of Urbino, and the juice was obtained as described above for "sloe".

Total anthocyanin content (TA)

The total anthocyanin content of the "sloe" and grape fruit juices was measured using the differential pH method reported by ELISIA *et al.* (2007) and TZULKER *et al.* (2007). Two aliquots of juice were dissolved separately in potassium chloride buffer (KCL, 0.025 M, pH 1.0) and sodium acetate ($CH_3CO_2Na.\ 3H_2O$, 0.4 M, pH 4.5) with a pre-determined dilution factor. The absorbancy measurements of the samples were read at 510 and 700 nm against a blank cell containing distilled and deionized water. The absorbance (A) of the diluted sample was then calculated as follows:

$$A = (A_{510nm} - A_{700\ nm})_{pH\ 1.0} - - (A_{510nm} - A_{700\ nm})_{pH\ 4.5}$$

The monomeric anthocyanin pigment concentration in the original sample was calculated according to the following formula:

$$\text{Anthocyanin content (mg/L)} = \frac{A \times MW \times DF \times 1000}{\varepsilon \times L}$$

where cyanidin-3-glucoside molecular weight (MW=449.2) and the molar ab-

sorptivity (ε = 26.900) constants were used.

Total polyphenol content

The total content of the polyphenolic compounds in fresh "sloe" and grape juices was determined by the Prussian Blue method described by HAGERMAN and BUTLER (1994) with slight modifications. Aliquots of the juice were made up to 1 mL with distilled water; after adding 60 μL of 0.1 M $FeNH_4(SO_4)_2$, they were incubated for 20 min at room temperature. Subsequently, 60 μL of 8 mM $K_3Fe(CN)_6$ were added to the sample, and after 20 min at room temperature the optical density of the mixture was determined at 720 nm (Jasco V-530 spectrophotometer, Tokyo, Japan). Quercetin was used as standard to construct a calibration curve.

DPPH assay

Radical scavenging activity was determined by a spectrophotometric method based on the reduction of an ethanol solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) (MELLORS and TAPPEL, 1966).

The DPPH assay was conducted as follows: a 100 μM DPPH ethanolic solution was prepared and 1.5 mL was added to the same volume of sample diluted in distilled water. The decreasing absorbance at 517 nm was recorded after 10 min and the percent decrease (corrected for the control, without addition of antioxidant agents) was taken as an index of the antioxidant capacity. Tests were carried out in triplicate.

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), BHT (butylated hydroxytoluene) and ascorbic acid were used as positive controls.

The IC_{50} values, defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%, were calculated from the results.

Lipoxygenase test

Inhibition of lipid peroxide formation was evaluated by the 5-lipoxygenase test in the sample and in the positive controls. The activity of the enzyme was assayed spectrophotometrically according to the method of HOLMAN, which was modified by SUD'INA *et al.* (1993).

The assay mixture (1 mL) contained: 10 mM linoleic acid, the sample (or the same quantity of solvent as reference) and 50 mM sodium phosphate, pH 6.8. This mixture was maintained at 20°C for 20 min.

Subsequently, 0.18 μg mL^{-1} of commercial 5-lipoxygenase was added to the mixture and the formation of hydroperoxides from linoleic acid was observed spectrophotometrically at 235 nm at 20°C.

The IC_{50} values, defined as the amount of antioxidant necessary to inhibit lipid peroxidation by 50%, were calculated from the results.

Oxygen Radical
Absorbance Capacity (ORAC)

The original method of CAO *et al.*(1993) was used with slight modifications. Fluorescein (3′,6′-dihydroxyspiro[isobenzofuran-1[3*H*],9′[9*H*]-xanthen]-3-one) was chosen as the fluorescent probe instead of B-phycoerythrin (B-PE) (OU *et al.*, 2001). When the sample with antioxidant activity had exhausted its capacity to trap the peroxyl radicals that had been induced by 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) at 37°C, fluorescein became the target of the radicals and lost its fluorescence. The area under the curve (AUC) of fluorescence decay was proportional to the antioxidant capacity of the sample, and a comparative evaluation with Trolox was performed.

The final reaction mixture for the assay (1 mL) was prepared as follows: 825 μL of 0.05 μM fluorescein sodium salt in 0.075 M sodium phosphate buffer,

pH 7.0, 100 μL of properly diluted sample in 0.075 M sodium phosphate buffer, pH 7.0 or 50 μM Trolox. The control was 0.075 M sodium phosphate buffer, pH 7.0. The fluorescence was read every 5 min at 37°C using a JASCO FP-6200 spectrofluorometer at 485 nm excitation, 520 nm emission. When stability was reached, the reaction was started with 75 μL of 5.55 mM AAPH and fluorescence was measured every 10 sec until zero fluorescence was detected.

The ORAC value is calculated according to the formula:

ORAC (μmol Trolox equivalent/g DW) = $[(A_S - A_B)/(A_T - A_B)]$ *kah*, where A_S is the AUC of fluorescein in the presence of the sample calculated with Spectra Manager for Windows 95/NT (Spectra Analysis) program, A_T is the AUC of the Trolox, A_B is the AUC of the control, *k* is the dilution factor, *a* is the concentration of Trolox in mmol/L and *h* is the ratio between the litres of extract and the grams of sample used for the extraction.

The contribution of individual anthocyanins to the antioxidant activity

Considering the total content of anthocyanidins found in the specimen was 55.13 mg/g DW and the ratios as reported in the literature, the following three anthocyanidins were relevant (DEINEKA *et al.*, 2005): cyanidin3-*o*-glucoside (11.4%), cyanidin3-*o*-rutinoside chloride (53.5%), peonidin3-*o*-rutinoside chloride (32.4%).

Respectively, the above ratios were used to determine the concentrations in our sample as follows:

cyanidin-3-*o*-glucoside	6.28 mg/g DW
cyanidin-3-*o*-rutinoside chloride	29.49 mg/g DW
peonidin-3-*o*-rutinoside chloride	17.86 mg/g DW

The above concentrations (mg/g) were then expressed as (mg/mL), based upon the DW content of prunus as (g/mL).

From the results obtained from the antioxidant dosages (DPPH, LIPOX, ORAC), which were obtained with the standard compounds (anthocyanidins), the antioxidant activity ascribed to the above-mentioned concentrations was estimated and the individual contribution was determined as the percentage of each anthocyanidin.

Statistical analysis

All the data are the average of a triplicate analysis. The data were recorded as mean ± standard deviation and analysed by SPSS (Version 9.0 for Windows 98, SPSS Inc.). One-way analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Duncan's multiple range test. The p values <0.05 were regarded as significant and those <0.01 very significant.

RESULTS AND DISCUSSION

In this study, the total polyphenol levels were determined both in the "sloe" juice and in the grape juice which has been shown to exert antioxidant activity (KEDAGE *et al.* 2007). The total polyphenol content was assessed as 83.54 mg/g DW and 1.38 mg/g DW for sloe juice and grape juice, respectively (Table 1). The total levels of anthocyanins assessed in the "sloe" juice and in the grape juice were 55.13 mg/g DW and 1.15 mg/g DW, respectively. Both the phenolic and anthocyanin contents determined in the *P. spinosa* juice were considerably higher than those of the grape juice whose values are quite similar to those detected in different cultivars of red grapes typical of southern Italy, known as Carigna-

Table 1 - Polyphenol and anthocyanin content of *Prunus spinosa* and *Vitis vinifera* juice. The results (± SD) are the means of 3 separate determinations.

	Prunus spinosa	*Vitis vinifera*
POLYPHENOLS		
mg/mL	154.12±30.71	2.55±0.19
mg/g DW	83.54±2.49	1.38±0.08
ANTHOCYANINS		
mg/mL	101.44±11.07	1.98±0.07
mg/g DW	55.13±5.64	1.15±0.08

no, Gaglioppo, Primitivo. The total content of polyphenols and anthocyanins in red grapes were dependent upon cultivar, and determined a different aptitude to wine production: a high concentration in polyphenolic substances could be added to the native grape varieties in order to produce wine with a more complex aroma (LOVINO *et al.*, 2006). KEDAGE *et al.* (2007) reported that the polyphenolic content of red and white grape varieties showed considerable differences and that a good correlation is found to exist between the total polyphenol content and their antioxidant power. These varieties of grapes, if consumed in adequate amounts, may confer health benefits, especially in populations prone to cardiovascular diseases (CVDs) (KEDAGE *et al.* 2007).

The results obtained showed that the juice from *P. spinosa* fruit has a significant antioxidant capacity compared with the activity of known antioxidants (ascorbic acid, BHT and Trolox). This was determined with three different acellular, *in vitro* tests, namely DPPH assay, 5-lipoxygenase assay and ORAC assay (Table 2).

The "sloe" fruit juice showed a good inhibition to lipid peroxidation. The value, expressed as IC_{50} in the 5-lipoxygenase assay (Table 2), is very similar to the value obtained with ascorbic acid and is comparable to the value obtained with Trolox, the elective standard for this assay.

In the reduction of the stable radical DPPH, the activity of the juice sample was comparable to ascorbic acid (IC_{50}= 0.16 and 0.11 mg/mL, respectively). The literature reports that anthocyanins such as cyanidin-3-*o*-glucoside and cyanidin-3-*o*-rutinoside contribute to the DPPH scavenging activity in the mature fruit. These two anthocyanins are well represented in "sloe" juice (OKI *et al.*, 2006).

Finally the antioxidant capacity of "sloe" fruit juice when measured by ORAC was 36.02 μmol Trolox equivalents/g of fruit (Table 2). These data are in accord with the ORAC values reported in the literature for small fruits such as vaccinium (blueberry, huckleberry, cranberry), rubus (raspberries, blackberries) and ribes (currants): the values obtained ranged from 19 to 131 μmol Trolox equivalents/g fruit for vaccinium, from 13 to 146 in rubus, and from 17 to 116 in ribes (MOYER *et al.*, 2002; ELISIA *et al.*, 2007). Different cultivars of four vaccinium species were analyzed for their antioxidant capacity which ranged from 13.9 to 45.9 μmol Trolox equivalents/g fruit (PRIOR *et al.*, 1998).

Table 2 - Antioxidant activity of *Prunus spinosa* juice. The results (± SD) are the means of 3 separate determinations.

	Lipoxygenase IC_{50} (μg/mL)	DPPH IC_{50} (mg/mL)	ORAC μmol eq. Trolox/g
Juice (*Prunus spinosa*)	18.00±1.97	0.160±1.1500	36.02±2.78
BHT	3.86±0.25	0.087±0.0120	
Trolox	11.89±1.22	0.007±0.0009	
Ascorbic acid	18.63±1.31	0.110±0.0073	

The data reported in the literature for other juices of fruits and vegetables and measured by ORAC assay showed that strawberry had the highest ORAC activity, followed by plum, orange, red grape, kiwi fruit, pink grapefruit, white grape, banana, apple, tomato, pear and melon with values ranging from 12.44 to 0.88 μmol Trolox equivalents/g fruits (WANG *et al.,* 1996). Consistently, it may be deduced that the antioxidant power of our sample (juice of *Prunus spinosa*) is comparable to the activity of small berries and is higher than several fruits and vegetables.

In many fruits and vegetables, the antioxidant activity can be attributed to the level of total polyphenols (SELLAPPAN *et al.,* 2002; RABABAH *et al.,* 2005; SOLOMON *et al.,* 2006). Polyphenols are comprised of several types of phytochemical compounds; one of the dominant classes in fruits are the anthocyanins that belong to the flavonoid group (RAPISARDA *et al.,* 1999). Anthocyanins, known for their antioxidant activity, are water-soluble pigments that are responsible for the red-blue color of many fruits (SEERAM *et al.*, 2001; NODA *et al.,* 2002).

TZULCHER *et al.* (2007) reported a positive correlation between the total polyphenol and anthocyanin contents and antioxidant activity of *Punica granatum* fruit juice.

Similarly, RANILLA *et al.* (2007) found a positive correlation between antioxidant activity and anthocyanin content in seed coats from Brazilian and Peruvian cultivars of *Phaseolus vulgaris*. The results reported indicate that polyphenols, and more specifically anthocyanins, contribute the major part of the antioxidant activity to some fruits.

A positive correlation has been established between the antioxidant activity and the polyphenol content of fruits (PRIOR *et al.*, 1998; KISELOVA *et al.,* 2006; KEDAGE *et al.,* 2007), suggesting that these compounds are responsible to a great extent for the activity. Furthermore, phenolic antioxidants contribute towards the protection of other antioxidants such as ascorbic acid (vitamin C), thus preventing its oxidative decomposition in fruit (MILLER and RICE-EVANS, 1997). Considering that VARDJAN *et al.* (1976) reported the content of ascorbic acid in *P. spinosa,* we may assume such interaction of compounds in our sample.

MONAGAS *et al.* (2006) observed that the ORAC values in grape skin were very close to the anthocyanin content. Once again this suggests that the antioxidant capacity was mainly due to the anthocyanins.

In accord with data reported in the literature for different fruits and vegetables, the total phenol and anthocyanin contents probably contributed a significant part of the antioxidant activity to "sloe" fruits, because *P. spinosa* fruit is rich in cyanidin-3-rutinoside (53.5%), peonidin-3-rutinoside (32.4%) and cyanidin-3 glucoside (11.4%) (DEINEKA *et al.*, 2005).

ELISIA *et al.* (2007) found that when 14 different anthocyanins were compared, cyanidin-3-glucoside showed a marked antioxidant efficacy *in vivo* and *in vitro.* Moreover anthocyanins have been shown to be novel antioxidants and potent inhibitors of lipid peroxidation when compared with other natural antioxidants (ZAFRA-STONE *et al.*, 2007).

Considering that anthocyanins contribute greatly to the antioxidant properties of "sloe" juice, the antioxidant capacity of "sloe" juice was compared to that of purified anthocyanins in order to calculate the extent of the antioxidant activity of these compounds.

The antioxidant activity of *P. spinosa* juice was evaluated by 5-lipoxygenase and DPPH assay and compared to the main anthocyanin standards (Figs. 1A and 1B) (DEINEKA *et al.,* 2005). The results shown in the graph are correlated to the concentration of each anthocyanin in "sloe" juice. The antioxidant activities of cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside and peonidin-3-O-rutinoside with respect to

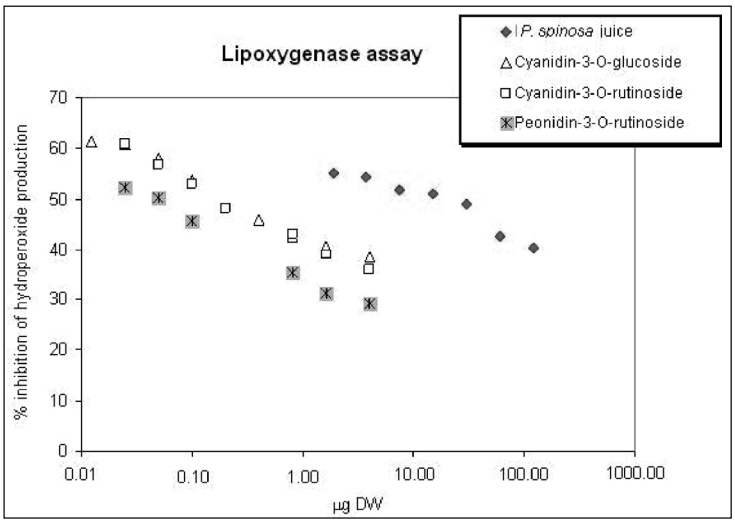


Fig. 1A - Antioxidant capacity of *P. spinosa* juice (◆), cyanidin-3-O-glucoside (Δ), cyanidin-3-O-rutinoside (□) and peonidin-3-O-glucoside (✳) in the lipoxygenase assay). Results represent the percentage of inhibition induced by increasing concentrations of sample on the hydroperoxide production and are the means S.E.M. from 3 separate determinations.

the total activity were calculated to be: 31.9, 28.0 and 25.1% (lipoxygenase assay) and 5.0, 38.6, 17.8% (DPPH assay), respectively.

Fig. 2 shows the same results obtained by the ORAC test; the antioxidant activity of "sloe" juice was compared to the three standard anthocyanins named above and to grape juice. The grape juice contained polyphenols and anthocyanidins (reported in Table 1). Before testing, the grape juice was adjusted so that it had the same total phenol concentration as that of the "sloe" juice. The two juices exhibited comparable activities, while cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside and peonidin-3-O-rutinoside were responsible for 11.5, 38.4 and 12.1%, respectively, of the total activity of "sloe" juice. The different results obtained with the three assays could depend on the different sensitivities and specificities of each method.

In conclusion, the data reported in this

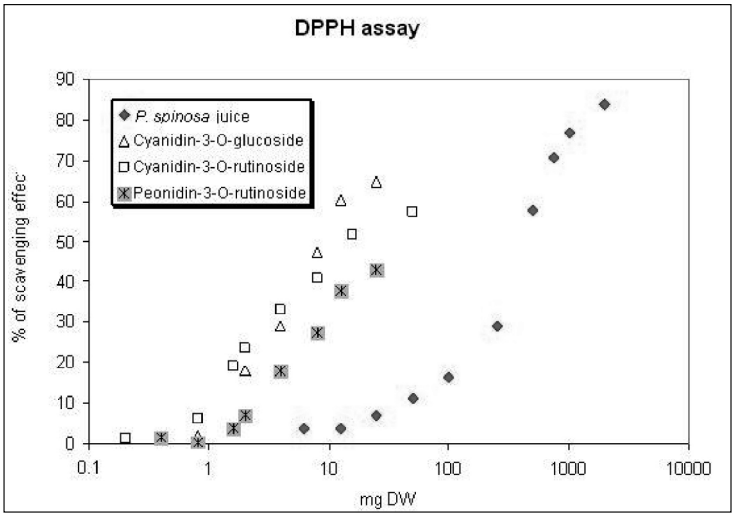


Fig. 1B - Antioxidant capacity of *P. spinosa* juice (◆), cyanidin-3-O-glucoside (Δ), cyanidin-3-O-rutinoside (□) and peonidin-3-O-glucoside (✳) in the DPPH assay. Results represent the percentage of inhibition induced by increasing concentrations of sample on the DPPH radicals, and are the means S.E.M. from 3 separate determinations.

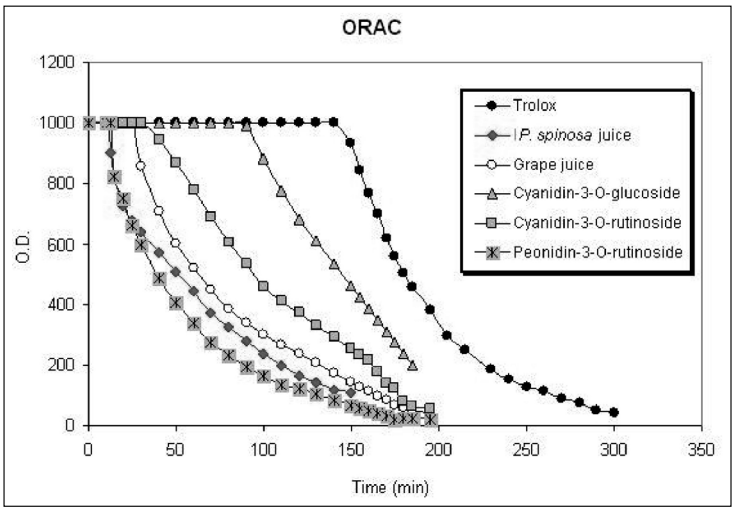


Fig. 2 - Fluorescence decay curves induced by AAPH of 5 μM Trolox (●), fresh juice of *P. spinosa* fruits (◆), grape juice (o), cyanidin-3-O-glucoside (Δ), cyanidin-3-O-rutinoside (□) and peonidin-3-O-glucoside (⊠).

paper could be of interest to the food industry particularly the sector involved in formulating dietary supplements that contain plant material as well as developing new ingredients with improved antioxidant properties from new plant sources such as *P. spinosa* fruit which is a spontaneous plant in the region of the Marches, but could be easily cultivated for more widespread use.

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SHORT COMMUNICATION

CHEMICAL COMPOSITION AND ANTIBACTERIAL ACTIVITY OF ESSENTIAL OILS OF BITTER FENNEL (*FOENICULUM VULGARE* MILL. VAR. *VULGARE*) AND DILL (*ANETHUM GRAVEOLENS* L.) AGAINST THE GROWTH OF FOOD-BORNE AND SEED-BORNE PATHOGENIC BACTERIA

EFFETTO DELLA COMPOSIZIONE CHIMICA ED ATTIVITÀ ANTIBATTERICA DI OLI ESSENZIALI DI FINOCCHIO AMARO (*FOENICULUM VULGARE* MILL. VAR. *VULGARE*) ED ANETO (*ANETHUM GRAVEOLENS* L.) SULLA CRESCITA DI BATTERI PATOGENI DI ORIGINE ALIMENTARE E DI ORIGINE SEMENTIERA

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ABSTRACT

In the present study, the chemical composition and antibacterial activity of essential oils of bitter fennel (*Foeniculum vulgare* Mill. var. *vulgare*) and

RIASSUNTO

Nel presente lavoro è stato studiato l'effetto della composizione chimica e l'attività antibatterica sulla crescita di batteri patogeni di origine alimentare e

- Key words: *Anethum graveolens* L., antibacterial activities, essential oils, *Foeniculum vulgare* Mill., plant pathogens -

dill (*Anethum graveolens* L.) were investigated against food-borne and seed-borne pathogenic bacteria. The chemical composition of bitter fennel and dill essential oils was analyzed by gas chromatography-mass spectroscopy. The major compounds found in the essential oils of bitter fennel and dill were estragole (37.6%) and limonene (33.1%), respectively. These essential oils have antibacterial activity against food-borne as well as seed-borne pathogenic bacteria. Fennel essential oil had the highest antibacterial activity against the food-borne bacterial pathogen *Staphylococus aureus*, while dill essential oil had the highest antibacterial activity against seed-borne pathogenic *Clavibacter michiganensis* subsp. *michiganensis*. Amongst all the bacterial species tested, the plant pathogenic agent *Pseudomonas syringae* pv. *tomato* had the greatest resistance to both essential oils. Considering the significant level of bacterial growth inhibition of seed-borne and food-borne pathogens, essential oils or their components could be promising seed disinfectants or food additives in appropriate products.

sementiera, di oli essenziali di finocchio amaro (*Foeniculum vulgare* Mill. var. *vulgare*) e di aneto (*Anethum graveolens* L.). La composizione chimica degli oli essenziali di finocchio amaro e di aneto è stata analizza per via gas-cromatografica accoppiata alla spettrometria di massa. I principali composti ritrovati negli oli essenziali di finocchio amaro e aneto erano rispettivamente l'estragolo (37,6%) e il limonene (33,1%). Questi oli essenziali presentano una attività antibatterica nei confronti dei batteri patogeni di origine alimentare e sementiera. L'olio essenziale di finocchio presentava l'attività antibatterica più alta nei confronti del batterio patogeno di origine alimentare *Staphylococus aureus*, mentre l'olio essenziale di aneto presentava l'attività antibatterica più alta nei confronti del patogeno di origine sementiera *Clavibacter michiganensis* subsp. *michiganensis*. Fra tutte la specie batteriche testate l'agente patogeno delle piante *Pseudomonas syringae* pv. *Tomato* presentava la maggiore resistenza nei confronti di entrambi gli oli essenziali. Considerando la significativa inibizione della crescita batterica dei patogeni di origine alimentare e sementiera, gli oli essenziali o i loro componenti potrebbero risultare, quando inseriti in appropriati prodotti, promettenti disinfettanti delle sementi o additivi alimentari.

INTRODUCTION

Food-borne bacterial disease agents are the cause of dangerous infections in humans. It has been estimated that as many as 30% of the people in developing countries suffer from food-borne diseases each year. Plant diseases caused by a range of bacteria are also one of the major problems in the cultivation of several crops. In many countries, quick and effective management of plant diseases and microbial contamination is generally achieved by using synthetic pesticides and antibiotics. Chemical control of plant diseases is based on the use of antibiotics (such as streptomycin) in the USA or copper compounds in the rest of

the world. These methods prevent bacterial multiplication, but are not always adequate for controlling seed-borne inoculum. Unfortunately, the frequent use of pesticides and antibiotics against plant and human pathogenic bacteria has led to the selection of bacterial populations that are resistant to antibiotics (URECH *et al.,* 1997). Factors including the high cost of pesticides, the development of pesticide-antibiotic-resistant food-borne and plant pathogenic isolates, governmental restrictions on the use of antibiotics against plant pathogens in the European countries, including Turkey, and concern for the environment highlight the need to find alternative control methods.

Secondary metabolites from aromatic and medicinal plants and essential oils have been used empirically for a wide variety of purposes for many centuries. Many of them have potential uses in medical procedures, and in the cosmetic, food preservation and pharmacological industries as well as for crop protection (HOSTETTMANN and WOLFENDER, 1997; RICE *et al.,* 1998). The antimicrobial properties of essential oils and their major constituents from a wide range of aromatic plant species have been assessed against the comprehensive range of microorganisms including bacteria, fungi and viruses (reviewed by JANSSEN *et al.,* 1987; DORMAN and DEANS, 2000; ISMAN, 2000; BURT, 2004; BAKKALI *et al.,* 2008). Bactericides of plant origin could be one approach to bacterial plant disease management because of their eco-friendly nature.

The Eastern Mediterranean region of Turkey has a flora that is rich in indigenous aromatic and medicinal plant species. Bitter fennel (*Foeniculum vulgare* Mill. var. *vulgare*) and dill (*Anetheum graveolens* L.), belong to the parsley family (Apiacaea). They have been widely grown in the Mediterranean basin of Turkey since antiquity and are known for their medicinal and aromatic properties. The seeds and essential oils of fennel are commonly used in the perfume industry, as a natural remedy against digestive disorders and to flavor foods and liquors. Dill plants are cultivated and the leafy tops are used for fresh consumption and food dressing. After flowering at the end of the harvest period, both plant species are left in the field for domestic animal consumption.

The objectives of this study were (a) to identify the chemical composition of the essential oils isolated from two related plant species, bitter fennel (*F. vulgare* var. *vulgare*) and dill (*A. graveolens*) grown in the region and (b) to investigate the *in vitro* antibacterial activities against ten bacterial species of Gram-positive and Gram-negative food-borne (*Salmonella* Thyphimirium, *Listeria monocytogenes, Escherichia coli* O157:H7, *Staphylococus aureus* and *Salmonella* Enteritidis) and seed-borne pathogenic bacteria (*Pseudomonas syringae* pv. *tomato*, *Xanthomonas axonopodis* pv. *vesicatoria*, *Erwinia carotovora*, *Clavibacter michiganensis* subsp. *michiganensis, Agrobacterium tumefaciens*).

MATERIALS AND METHODS

Plant material and extraction of essential oils

The plants used in this study were identified and a voucher specimen was deposited in the herbarium of the Plant Protection Department of Mustafa Kemal University (No. FvAu2 and AgSc4). For the extraction of essential oils, dill plants and fennel seeds were collected in the Samandağ (36° 16' N; 35° 48' E, 38 m) and Narlıca (36° 14' N; 36° 13' E, 104 m) districts, respectively, in the Eastern Mediterranean region of Turkey. The annual average temperatures in Samandağ and Narlıca were 20.1° and 18.1°C; rainfall was 750 and 1,124 mm, respectively. The characteristic soils of

Samandağ and Narlıca are aquic xerofluent and calcixerept, respectively. The leaves and stems of the dill plants were used for the extraction of the essential oils, while the seeds were used for the extraction of the fennel essential oil. Three different lots of air-dried plant material were used separately for extraction. For each lot, the air-dried plant material (200 g) was placed in a 5 L round-bottom distillation flask and 3 L of double-distilled water were added. The essential oils were obtained by steam distillation using a Clevenger-type apparatus (Ildam, Ankara, Turkey) for 3 h. The oils were separated, dried over anhydrous sodium sulphate and stored in an amber bottle at 4°C until used.

GC-MS analysis of the essential oil

The essential oil analysis was performed using a Hewlett-Packard 6890 (Waldbronn, Germany) GC linked to a Hewlett-Packard 5973 mass selective detector equipped with a HP-5 MS (Crosslinked 5% Phenyl Methyl Siloxane) capillary column (30 m x 0.25 mm i.d., 0.25 μm film thickness). The carrier gas was helium, at a ratio of 1.0 mL min^{-1}. The amount of the sample injected was 0.1 μL in split mode (50:1). The initial oven temperature was 50°C, increased at a rate of 2°C min^{-1} to 90°C, 5°C min^{-1} to 210°C and finally isothermal for 5 min. The injector and detector temperatures were maintained at 250° and 280°C, respectively. The quadrupole mass spectrometer was scanned over the 50-550 amu range at 1.53 scan s^{-1}, with an ionizing voltage of 70 eV. The major components of the essential oils were identified by comparing the retention indices and mass spectra with those of authentic samples or published data (ADAMS, 2001). They were then matched with the Wiley275.L Registry of Mass Spectral Data (MCLAFFERTY, 1994). The retention indices were calculated for all of the volatile constituents using a homologous series of *n*-alkanes C_8-C_{20}.

Test microorganisms and culture methods

The food-borne and human pathogenic bacteria used were *Escherichia coli* O157:H7 (ATCC 35218), *Salmonella typhimurium* (RHSM 1996), *Listeria monocytogenes* (NCTC 2167), *Staphylococcus aureus* (ATCC 43300) and *Salmonella enteritidis* (OSU 799). Stock cultures were maintained at 4°C on Tryptic Soy Broth (Merck) amended with 5 g L^{-1} Yeast Extract (Merck, Darmstadt, Germany) and 15 g L^{-1} agar agar (Merck, Darmstadt, Germany). Cultures of the plant pathogenic bacterial isolates of *Pseudomonas syringae* pv. *tomato* (*Pst*Hd1), *Xanthomonas axonopodis* pv. *vesicatoria* (*Xav*Md3), *Erwinia carotovora* pv. *carotovora* (*Ecc*Md17), *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*Ad12) and *Agrobacterium tumefaciens* (*At*Ad2) used in the study were isolated from infected leaves, fruit and stems of tomato and pepper plants growing in the region. They were identified on the basis of fatty acid methyl ester (FAME) analysis as described by SASSER (1990). *Pst* was grown and maintained on King's medium B (KB) agar (Merck, Darmstadt, Germany), while other isolates (*Xav, Cmm, Ecc* and *At*) were grown on yeast glucose chalk (YDC) agar. Stock cultures were maintained on appropriate media at 4°C and sub-cultured once a month.

Active cultures were prepared for the experiments by transferring a loopful of cells from the stock cultures to 10 mL flasks of appropriate nutrient broth which were then incubated for 24 h at 37°C (food-borne pathogen) or 26°C (plant pathogen). The cultures were diluted with an appropriate fresh broth to achieve optical densities corresponding to 10^8 CFU mL^{-1}. These suspensions were used as required.

Determination of antibacterial activity of the essential oil

The antibacterial activity of the essential oils was determined by using the paper disc diffusion technique. Briefly, the test was performed in sterile Petri dishes (100 mm diameter) containing an appropriate solid and sterile media. The surface of the plates was inoculated with 200 μL of bacterial suspension prepared as described previously. Sterile filter paper (Whatman No. 1) discs (6 mm in diameter) containing 10 μL of the tested essential oils were placed in the centre of the agar surface. A disc containing the 10 μL of sterile broth media was used as the negative control. Two different reference antibiotic, rifampicin and tetracycline, (Sigma, Taufkirchen, Germany) amended discs, at 100 μg mL^{-1} concentrations, were used as the positive control for comparison. Each individual Petri dish was immediately covered to prevent eventual evaporation. After allowing the essential oils to diffuse across the surface for 1 h at room temperatures, the plates were sealed with sterile parafilm and incubated at 26°C (for plant pathogens) or 37°C (for foodborne pathogens) for 48 h. The antibacterial activities of the oils and antibiotics were demonstrated by a clear zone of inhibition around the disc. The zone of inhibition was measured using Vernier calipers.

Statistical analysis

All experiments were performed twice using five replicate plates of each essential oil for each bacterium. The data were subjected to analysis of variance (ANOVA) using the SPSS statistics program (Version 11.5). The significance between treatments was determined using Tukey's Test ($P \leq 0.05$). The data from two independent experiments were analyzed separately but were not significantly different ($P > 0.05$).

RESULTS AND DISCUSSIONS

The average essential oil yields of bitter fennel and dill were about 7.9 and 0.9% (v/w), respectively. The qualitative and quantitative analyses of essential oil constituents were identified by GC-MS and the results are reported in Table 1, together with their relative percentages in the order of elution from the column. The bitter fennel essential oil is made up of 13 constituents, which account for the total amount. The major component was estragole (37.6%), followed by limonene (35.4%), fenchone (12.8%) and carvone (3.4%). The composition of the bitter fennel oil analysed was typical of the variety of *Foeniculum vulgare* Mill. var. *vulgare* (bitter fennel) (BARAZANI *et al.*, 2002). While some variations were observed, the composition of the bitter fennel essential oil was generally in agreement with that reported from different countries.

The essential oil of dill had a very different chemical composition with a total of 16 constituents, which accounted for 99.1% of the total amount. According to GC-MS analysis, limonene was the most abundant volatile compound, comprising 33.1% of the total volatiles, followed by α-phellandrene (24.9%), carvone (16.8%), dillapiole (9.6%), *p*-cymene (3.7%) and α-pinene (3.0%). The properties of dill oil depend largely on the proportions of limonene, carvone and α-phellandrene; the characteristics of the essential oil predominated if the carvone content was less than 35%. The aroma of dill herb oil from the leaves and stems is due to phellandrene and dill ether. Minor components like apiole or myristicin may be typical of some varieties which allow the chemotypes to be distinguished (KRÜGER and HAMMER 1996). Notable quantities of apiole were detected in this study. Myristicin accounted for 1.38% of the total oil. Quantitative differences in the essential oil components in *F. vulgare* and *A. graveolens* from dif-

Table 1 - Chemical constituents of essential oils of bitter fennel (*F. vulgare* var. *vulgare*) and dill (*A. graveolens*). The compounds are listed in order of elution time from a HP-5MS column.

No.	Compounds	RI[a]	Fennel % MS[b]	Dill % MS[b]
1	α-Pinene	935	2.6±0.03	3.0±0.08
2	Sabinene	971	1.5±0.02	0.4±0.03
3	Myrcene	990	0.8±0.02	0.5±0.02
4	α-Phellandrene	1002	0.4±0.03	24.9±0.3
5	*p*-Cymene	1021	-	3.7±0.25
6	Limonene	1025	35.4±0.69	33.1±0.17
7	*trans*-β-Ocimene	1035	2.1±0.02	-
8	γ-Terpinene	1060	0.4±0.02	0.4±0.03
9	Fenchone	1081	12.8±0.26	-
10	Terpinen-4-ol	1180	-	0.2±0.01
11	Dill ether	1187	-	0.6±0.03
12	*cis*-Dihydrocarvone	1191	-	1.1±0.07
13	Estragole	1193	37.6±0.59	-
14	*trans*-Dihydrocarvone	1197	-	1.7±0.02
15	Carvone	1242	3.4±0.05	16.8±0.24
16	*p*-Anisaldehyde	1250	0.3±0.02	-
17	*trans*-Anethole	1286	2.0±0.07	-
18	β-Caryophyllene	1417	-	0.4±0.03
19	Germacrene D	1477	0.1±0.01	0.9±0.04
20	Myristcin	1518	-	1.8±0.05
21	Dillapiole	1623	-	9.6±0.04

[a] The retention index was calculated using a homologous series of n-alkenes C_8-C_{20}.
[b] The values (±SE) are expressed as means of three different determinations and were obtained from electronic integration measurements using selective mass detector.
Dash indicates the compound was not found.

ferent countries indicate that environmental and genetic factors strongly influence its chemical composition (MAROTTI *et al.*, 1994).

The antibacterial activities of the essential oils measured by the agar disc diffusion method are given in Table 2. The essential oils isolated from both plant species showed varying levels of antibacterial activities and significant differences in microbial susceptibility. The essential oil of *F. vulgare* showed the highest and lowest activities against food-borne bacterial pathogens *S. aereus* (13.4 mm) and *P. syringae* pv. *tomato* (6.5 mm), respectively. The essential oil of *A. graveolens* had the highest activity against gram-positive plant pathogenic bacterial agent *C. michiganensis* subsp. *michiganensis* (19.4 mm). Gram-negative bacterial agent *P. syringae* pv. *syringae* was the most resistant bacterial species to the dill essential oil just like fennel (6.3 mm). Volatile compounds from plants, especially essential oils, have antimicrobial activity against a variety of food-borne, human and plant pathogens (BAKKALI *et al.*, 2008). A relatively limited number of reports were found in the literature regarding plant extracts and/or essential oils against phytopathogenic bacteria. To our knowledge no research has been conducted to evaluate the efficacy of the essential oils used in this study against plant pathogenic bacteria. The results of this study confirm that es-

sential oils from the Turkish specimens of *F. vulgare* and *A. graveolens* have antibacterial activity against food-borne and economically important seed-borne plant pathogenic bacteria. Similar antibacterial activities of essential oils and/or major components of dill and fennel plants from different countries against a variety of food-borne and human pathogens have been reported (AGGRAWAL *et al.,* 2002; DELAQUIS *et al.,* 2002; DADALIOGLU and EVRENDILEK, 2004; OZKAN *et al.,* 2003; RUBERTO *et al.,* 2000; SINGH *et al.,* 2005; SCHELZ *et al.,* 2006). On the other hand, very few studies have shown the antibacterial activities of essential oils against plant pathogenic bacteria (SATISH *et al.,* 1999; LO CANTORE *et al.,* 2004).

The chemical methods of plant disease management are expensive and can damage the beneficial microbial population present in the ecosystem. The obvious pollution problems that result from indiscriminate use of synthetic pesticides and their toxic effect on non-target organisms have prompted investigations on the use of pesticides of plant origin. Natural plant products are important sources of new agrochemicals for the control of plant diseases. Furthermore, biocides of plant origin are non-phytotoxic, systemic and easily biodegradable (BAKKALI *et al.,* 2008).

Among the bacteria, six species showed similar sensitivity to both essential oils, whereas four bacteria showed differences in the sensitivity to fennel and dill oils. The size of the inhibition zone recorded for *A. tumefaciens*, *C. michiganensis* subsp. *michiganensis*, and *L. monocytogenes* in Petri dishes with fennel oil added was significantly less than that recorded in the Petri dishes with dill oil added (Table 2). The size of the inhibition zone recorded for *Staphylococus aureus* was significantly greater in fennel oil-added Petri dishes than that in the dill oil-added Petri dishes. The mode of action of essential oils against bacterium is unclear. The essential oil and its components may disrupt the cell membrane of the fungal and bacterial pathogen and change its permeability (COX *et al.,* 2000). Given the broad spectrum activities of fennel and dill essential oils and the general membrane-damaging effects of essential oils, this

Table 2 - Antibacterial activities of bitter fennel (*F. vulgare* var. *vulgare*) and dill (*A. graveolens*) essential oils against plant pathogenic and food-borne bacterial species.

Microorganisms	Inhibition zone (mm)[a]			
	Bitter Fennel	Dill	Tetracycline	Rifampicin
Pseudomonas syringae pv. *tomato*	6.5 aA	6.3 aA	20.6 bC	15.8 bB
Erwinia carotovora pv. *carotovora*	6.9 abA	6.78 aA	41.4 cdC	21.1 cB
Xanthomonas axonopodis pv. *vesicatoria*	10.9 efA	12.9 eA	42.6 dB	49.7 eC
Agrobacterium tumefaciens	8.5 cdA	10.4 cdB	50.7 eD	29.6 dC
Clavibacter michiganensis subsp. *michiganensis*	9.6 deA	19.4 fB	18.7 bB	53.0 fC
Listeria monocytogenes	7.4 abcA	10.1 cdB	39.6 cD	12.4 aC
Staphylococcus aureus	13.37 gC	10.8 dB	8.4 aA	12.6 aC
Escherichia coli	11.8 fB	11.4 deB	8.3 aA	16.6 bC
Salmonella Enteritidis	8.1 bcA	8.8 bcA	9.4 aAB	16.5 bC
Salmonella Thyphimirium	8.6 cdA	8.4 bA	9.6 aB	11.6 aC

[a] Includes diameter of disc (6 mm). Mean values (n=5) followed by the same small or capital letters within the column or row, respectively, are not significantly different according to Tukey's test ($P \leq 0.05$).

variability is probably due to the rate at which their active components diffuse through the cell wall into the phospholipid regions of the cell membrane structure as suggested by COX *et al.* (2000).

In addition to their antibacterial activities, the *F. vulgare* essential oil show antiviral activity that has inhibitory properties against PVX (potato virus X), TMV (tobacco mosaic virus) and TRSV (tobacco ring spot virus). The essential oil at a concentration of 3000 ppm totally inhibited the formation of local lesions (SHUKLA *et al.,* 1989). Similar studies were conducted previously to assess the efficacy of essential oils from medicinal plants, including sweet fennel, which is rich in *trans*-anethole, against phytopathogenic fungi (SOYLU *et al.,* 2006; 2007).

In summary, it can be concluded that the essential oils of bitter fennel and dill, rich in estragole (37.6%) and limonene (33.1%), respectively, possess significant antibacterial activities against not only food-borne, but also seed-borne pathogenic bacterial agents. Copper-based preparations are generally applied to fight against plant pathogenic bacterial agents using foliar spraying, which only controls leaf symptoms. However, the development of fungicide resistance requires that new fungicides be developed that have different modes of action. Since essential oils and/or their main components have been reported to possess fungicidal and bactericidal activities, the treatment of seeds with essential oils or their components could be used as a seed disinfectant against seed-borne plant bacterial agents. However further experiments are needed to obtain information about the economic aspects and antibacterial activities of essential oils *in vivo* and determine if there are phytotoxic effects on seed germination. It is also likely that these essential oils could be used as antimicrobial agents to prevent the spoilage of food products.

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SHORT COMMUNICATION

INFLUENCE OF *SPIRULINA PLATENSIS* BIOMASS ON MICROBIOLOGICAL VIABILITY IN TRADITIONAL AND PROBIOTIC YOGURTS DURING REFRIGERATED STORAGE

INFLUENZA DELLA BIOMASSA DI SPIRULINA SULLA VITALITÀ MICROBIOLOGICA IN YOGURTS TRADIZIONALI E PROBIOTICI DURANTE LO STOCCAGGIO REFRIGERATO

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ABSTRACT

The effect of adding *Spirulina platensis* on the viability of a yogurt starter and *Bifidobacterium animalis* was assessed during 28 days of refrigerated storage. Four types of yogurts were manufactured: The control yogurt; yogurt containing *S. platensis*; yogurt containing *B. animalis*; yogurt containing *S. platensis* and *B. animalis*. On average, the survival rate of *Streptococcus salivarius* ssp. *thermophilus* was better than those of both *Lactobacillus del-*

RIASSUNTO

È stato valutato l'effetto dell'aggiunta di *Spirulina platensis* sulla vitalità di uno yogurt starter e sul *Bifidobacterium animalis* durante 28 giorni di stoccaggio refrigerato. Sono state prodotte quattro tipologie di yogurt: yogurt controllo, yogurt contenente *S. platensis*; yogurt contenente *B. animalis*; yogurt contenente *S. platensis* e *B. animalis*. In media il tasso di sopravvivenza dello *Streptococcus salivarius* ssp. *thermophilus* risultava migliore sia di quello

- Key words: microbiological stability, probiotic bacteria, *Spirulina platensis*, viability, yogurt -

brueckii ssp. *bulgaricus* and *B. animalis*. *S. platensis* biomass significantly decreased the viability of *S. thermophilus* but increased the viable cell counts of *L. bulgaricus* in both traditional and probiotic yogurts. The stability of *B. animalis*, on average, remained statistically constant throughout the refrigerated storage period in probiotic algal yogurt compared to probiotic non-algal yogurt. It was also shown that probiotic algal yogurt has a shelf-life of one month with a viable *Bifidobacterium* cell count of at least 6 log cfu g^{-1}.

del *Lactobacillus delbrueckii* ssp. *bulgaricus* sia del *B. animalis*. La biomassa della *S. platensis* riduceva significativamente la vitalità dello *S. thermophilus* ma incrementava la conta vitale del *L. bulgaricus*, negli yogurts tradizionali e probiotici. La stabilità del *B. animalis*, in media, restava statisticamente costante durante il periodo di stoccaggio refrigerato nello yogurt probiotico algale rispetto al probiotico non-algale. È stato inoltre dimostrato che lo yogurt probiotico algale presentava una shelf-life di un mese con la conta vitale del *Bifidobacterium* di almeno 6 log cfu g^{-1}.

INTRODUCTION

Yogurt is a common dairy product that is consumed worldwide. The addition of probiotic bacteria to yogurt improves its functionality and health benefits. Within the various probiotic organisms, the genus *Bifidobacterium* has been extensively studied in fermented dairy products and the health benefits derived from the consumption of bifidobacteria have been well documented (de ROSS and KATAN, 2000; ROLFE, 2000).

A number of studies have demonstrated that the viability of bifidobacteria incorporated in yogurt is often poor and that more attention should be given to accurately identifying and describing the safety and functionality of these microorganisms (HAMILTON-MILLER *et al.*, 1999; TEMMERMAN *et al.*, 2003). A total of 58 probiotic products obtained worldwide, which claimed to contain *Bifidobacterium* strains (including 22 yogurts) were investigated; *Bifidobacterium animalis* ssp. *lactis* was the most frequently found species (MASCO *et al.*, 2005). JAYAMANNE and ADAMS (2006) investigated the level, species and resistance to acidity and oxidative stress of common *Bifidobacterium* ssp. found in bio-yogurts in the UK. They reported that *Bifidobacterium animalis* ssp. *lactis*, the only *Bifidobacterium* species found in bio-yogurts, were highly resistant to acidity and oxidative stress.

Indigenous populations have used microalgals for centuries. The growing demand for natural additives for healthy foods has increased interest in microalgal biotechnology over the last two decades (JIMÉNEZ *et al.*, 2003; DUFOSSÉ *et al.*, 2005; SPOLAORE *et al.*, 2006). The marine world is a relatively untapped reservior of bioactive ingredients that could have numerous applications in food processing, storage and fortification (RASMUSSEN and MORRISEY, 2007).

Cyanobacteria or blue-green algae are photoautotrophic microorganisms that are widely distributed in nature (PARADA *et al.*, 1998; MOLNAR *et al.*, 2005). *Spirulina* is the best known genus because of its high protein content and excellent nutritional value. *Spirulina* has been reported to have various health-promoting effects; it alle-

viates hyperlipidemis, reduces hypertension, protects against renal failure, promotes the growth of intestinal *Lactobacillus*, and reduces elevated serum glucose levels. The chemical composition includes essential amino acids, vitamins, natural pigments, and essential fatty acids, particularly α-linolenic acid, a precursor of prostaglandins (de CAIRE *et al.*, 2000; PARADA *et al.*, 1998; SHIMAMATSU, 2004; SPOLAORE *et al.*, 2006). It has also been reported that *Spirulina* has antimicrobial activity (MENDIOLA *et al.*, 2007).

S. platensis has been shown to promote the growth of lactic acid bacteria in synthetic media (PARADA *et al.*, 1998), milk and fermented milk (VARGA and SZIGETI, 1998; de CAIRE *et al.*, 2000; VARGA *et al.*, 2002). However, the effect of *S. platensis* on the viability of starter bacteria and *B. animalis* in yogurt has not been studied. The objective of this study was to investigate the effect of *S. platensis* on the growth and viability of yogurt starter bacteria and *B. animalis* in the presence of yogurt bacteria during 28 days of refrigerated storage at 4°C.

MATERIALS AND METHODS

Strains and ingredients

A commercial yogurt starter culture containing *Streptococcus salivarius* ssp. *thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* (YC X-11) and *B. animalis* (Bb-12) (Chr. Hansen A/S, Hørsholm, Denmark) was used in freeze-dried direct vat set (DVS) form containing 10 E-11 and 10 E-10, respectively, and stored according to the manufacturer's recommendations.

Skim milk powder was provided by Pınar Dairy Products (İzmir, Turkey).

S. platensis (Ege-Macc 31) was obtained from the culture collection of Ege University Microalgae Culture Collection (Ege-Macc, Turkey).

Culture conditions for *Spirulina platensis* biomass

S. platensis was cultured in Zarrouk's medium (FOX, 1996) in outdoor paddle-wheel circulating ponds in a greenhouse. The day-night temperatures were approximately 28°-30°C and 25°-27°C, respectively. The culture was illuminated by daylight and had a pH of 9.5. The culture was harvested in the logarithmic phase by filtration with a plankton net (30 μm mesh). The paste was squeezed in the plankton net to remove the culture medium. The biomass was dried at 27°C for 3 h. The dried *S. platensis* biomass contained 68% protein, 1.5% lipid, 21% carbohydrate, and 7% ash.

Yogurt preparation

Yogurts (set style) were made from cow milk having 3.1% (W/V) milk fat, 3.04% (W/V) protein and 0.70% (W/V) ash. Skim milk powder (3.5%) was added to raw milk at 20°C. Raw milk was treated at 85°C for 30 min, then cooled to 45°C and divided into four lots. All mixtures were then inoculated with yogurt starter culture. The first lot with no supplements or probiotic bacteria was used as the control. *S. platensis* biomass (3 g L^{-1}) was added to the second lot, while *B. animalis* was added to the third lot. The fourth lot contained both *S. platensis* biomass and *B. animalis*. All cultures were used according to the manufacturer's instructions. Yogurt starter culture was poured into sterilized milk (1 L) at 40°C and mixed thoroughly; 4 mL of the mixture were then added. *B. animalis* (0.01%) was also added. The mixtures were poured into 100 mL plastic cups and incubated at 40°C for about 4 h until the pH was 4.7. After fermentation, yogurt samples were cooled and transferred to a refrigerator at 4°C and stored at this temperature for 28 days during which time cell counts were taken. All yogurt samples were uniformly

mixed and samples were taken aseptically for microbiological analyses.

Chemical analyses

Total solids, protein and ash contents were determined by the AOAC methods (1999), while the fat contents were analyzed by the Gerber method (RENNER, 1993). The pH values of the yogurt samples were measured at 17°-20°C using a pH meter (Hanna Instruments Model pH:211, Woonsocket, RI, USA).

Microbiological analyses

One gram of each yogurt sample was diluted with 9 mL of sterile 0.1% (W/V) peptone water (Oxoid, Hampshire, UK) and mixed uniformly with a vortex mixer. Subsequent serial dilutions were made and the viable yogurt starter and *B. animalis* counts were taken using the pour plate technique. The counts of *S. thermophilus* were taken on M-17 agar (Merck, Darmstadt, Germany) after incubating the plates aerobically at 37°C for 72 h (LUCAS *et al.*, 2004). MRS agar (Merck, Darmstadt, Germany) adjusted to pH 5.2, and anaerobic incubation at 42°C for 72 h was used for the *L. bulgaricus* counts (SHAH, 2000). *B. animalis* was counted according to the method of LANKAPUTHRA *et al.* (1996) using MRS-NNLP (nalidixic acid, neomycin sulphate, lithium chloride and paramomycin sulphate) agar. The inoculated plates were incubated anaerobically at 37°C for 72 h using an oxygen-free environment and a CO_2 atmosphere in anaerobic jars (Merck, Darmstadt, Germany). Plates containing 25 to 250 colonies were counted and recorded as colony forming units (cfu) per gram of sample.

Statistical analyses

Each experiment was independently replicated twice in a completely randomized design. All analyses and counts were done in duplicate.

Analysis of variance (ANOVA) was applied on chemical and microbiological data using the SPSS programme. Differences among means were tested for significance using Duncan's multiple range test. The level of significance was set at 95% (SPSS, 1997).

RESULTS AND DISCUSSION

Table 1 shows the mean values for total solids, protein and ash contents and pH of the yogurt samples on the first day.

The pH values of yogurt samples B and D decreased when *S. platensis* was added compared to those of the other samples ($p<0.05$). This decline was probably due to the fact that the *S. platensis* bio-

Table 1 - Total solids, milk fat, protein and ash content and pH values of yogurt samples at the first day (mean values±standard deviations, n=2)*.

Product	Total solids (%)	pH	Protein (%)	Milk fat (%)	Ash (%)
A	14.69±0.06^{a}	4.65±0.00^{b}	3.95±0.08^{b}	3.05±0.02^{a}	0.89±0.01^{c}
B	14.78±0.02^{a}	4.50±0.00^{c}	4.43±0.00^{a}	3.00±0.00^{a}	0.90±0.01^{c}
C	14.66±0.00^{a}	4.70±0.00^{a}	4.06±0.02^{b}	3.05±0.02^{a}	0.99±0.01^{b}
D	14.68±0.06^{a}	4.50±0.00^{c}	4.32±0.00^{a}	3.00±0.00^{a}	1.05±0.01^{a}

Yogurts A, B, C and D contain no supplement, *S. platensis*, *B. animalis* and *S. platensis* and *B. animalis*, respectively.
$^{a-c}$ Means with different letters in the same column are different ($p<0.05$).
* Total solids, milk fat, protein and ash values were measured as W/V.

Table 2 - Changes in the viable counts of *S. thermophilus*, *L. bulgaricus* and *B. animalis* during refrigerated storage of yogurt samples (log cfu g^{-1}).

Product	Storage days					Mean of storage
	1	7	14	21	28	
			S. thermophilus			
A	8.90±0.09^{aA}	8.75±0.05^{abAB}	8.76±0.02^{aAB}	8.86±0.06^{aA}	8.67±0.07^{abB}	8.78±0.10^{a}
B	8.51±0.02^{bAB}	8.64±0.00^{bA}	8.28±0.14^{cBC}	8.42±0.06^{cABC}	8.18±0.20^{cC}	8.41±0.19^{c}
C	8.80±0.00^{aB}	8.79±0.06^{aB}	8.77±0.04^{aB}	8.72±0.00^{abB}	8.91±0.01^{aA}	8.80±0.07^{a}
D	8.49±0.04^{bA}	8.43±0.03^{cA}	8.52±0.10^{bA}	8.58±0.06^{bA}	8.50±0.02^{bA}	8.50±0.07^{b}
			L. delbrueckii ssp. *bulgaricus*			
A	5.64±0.05^{bA}	5.79±0.05^{cA}	4.54±0.08^{bC}	5.29±0.13^{cB}	5.25±0.07^{bB}	5.30±0.46^{d}
B	5.83±0.00^{bA}	5.51±0.04^{dB}	5.72±0.00^{aA}	5.77±0.06^{bA}	5.48±0.11^{bB}	5.66±0.16^{c}
C	5.60±0.42^{bB}	6.86±0.01^{bA}	5.90±0.02^{aB}	5.89±0.06^{bB}	6.83±0.18^{aA}	6.21±0.57^{b}
D	6.58±0.00^{aC}	7.21±0.03^{aA}	5.45±0.02^{aE}	6.22±0.06^{aD}	6.78±0.01^{aB}	6.45±0.63^{a}
			B. animalis			
C	7.04±0.02^{B}	6.30±0.00^{C}	6.39±0.13^{C}	7.52±0.01^{A}	6.45±0.21^{C}	6.74±0.50^{a}
D	6.44±0.06^{B}	7.03±0.00^{A}	6.82±0.31^{AB}	7.00±0.00^{A}	6.73±0.18^{AB}	6.80±0.26^{a}

Each value is a mean of two trials, and each trial was examined in duplicate. Data are presented as mean values ± standard deviation.
Yogurts A, B, C and D contain no supplement, *S. platensis*, *B. animalis* and *S. platensis* and *B. animalis*, respectively.
$^{a-d}$ Means in the same column with different small letters are significantly different (p<0.05).
$^{A-E}$ Means in the same row with different capital letters are significantly different (p<0.05).

mass stimulated the growth of *L. bulgaricus*. The viable cell counts of *L. bulgaricus* were higher in algal yogurts on the first day of storage. VARGA and SZIGETI (1998) also reported pH values that were lower in algal yogurt containing dried *S. platensis* biomass than those of natural yogurt at the beginning of storage. Similarly, the pH values in milk inoculated with the mixed culture of *S. thermophilus* and *L. bulgaricus* and containing *S. platensis* decreased more rapidly than those in the control samples during the first 3 h of fermentation (VARGA *et al.*, 1999a). VARGA *et al.* (2002) also reported a more marked decrease in pH value in the *S. platensis*-supplemented fermented ABT milk than in the control ABT milk during storage.

S. platensis-supplemented yogurts had higher protein contents than the yogurts with no supplements (p<0.05). This was due to the high protein content of the microalgae *S. platensis* which was also reported by PELIZER *et al.* (2003).

The changes in the viable counts of *S. thermophilus*, *L. bulgaricus* and *B. animalis* in the yogurt samples during refrigerated storage are reported in Table 2. There were significant differences in the viability of *S. thermophilus* between the yogurt samples with or without *S. platensis*. In general, higher viable counts of *S. thermophilus* were obtained in yogurts without *S. platensis* (p<0.05).

In contrast to the present results, de CAIRE *et al.* (2000) reported that *S. platensis* promoted the growth of *S. thermophilus* in milk. Similarly, *S. platensis*-supplemented ABT fermented milk contained higher (p<0.05) *S. thermophilus* counts than the control ABT fermented milk after 6 weeks of refrigerated storage (VARGA *et al.*, 2002).

The viable counts of *S. thermophilus* in all the yogurt samples were above 8

log cfu g^{-1} during the 28 d storage period. VARGA and SZIGETI (1998) also counted a minimum of 8 log cfu g^{-1} of viable *S. thermophilus* in both natural and algal yogurts during storage at 4°C.

Significant ($p<0.05$) decreases (approximately 0.2-0.3 log cycles), in the survival rates of *S. thermophilus* were observed in yogurts A and B after 28 days of storage. Similar results were obtained in the control and algal ABT fermented milk at the end of the storage period (VARGA *et al.*, 2002). On the other hand, the viable counts of *S. thermophilus* did not change significantly in sample D during the storage time ($p<0.05$). On average, the survival rate of *S. thermophilus* was better than that of both *L. bulgaricus* and *B. animalis*. The viable counts of *S. thermophilus* were 2-3 log higher than those for *L. bulgaricus* in the yogurt samples. These observations are in line with those of VINDEROLA *et al.* (2000) and AKALIN *et al.* (2004).

According to the mean storage values, supplementation with *S. platensis* significantly increased the viable *L. bulgaricus* counts when compared to those of yogurts containing no algae ($p<0.05$). Supplementation with algae biomass significantly increased the viable *L. bulgaricus* counts in both the natural and probiotic yogurts ($p<0.05$). This effect can be attributed to the presence of free amino acids, peptone, adenine and hypoxanthine in the algal biomass. These nitrogenous substances are capable of significantly ($p<0.05$) stimulating the growth and acid production of *L. bulgaricus* (VARGA *et al.*, 1999b; MOLNAR *et al.*, 2005). VARGA and SZIGETI (1998) observed that *S. platensis* promotes the growth of *L. bulgaricus* in yogurt. They reported *L. bulgaricus* counts above 7 log cycle while in the present study the counts were below 6 log cycle in the treatments A and B. The addition of *S. platensis* also increased the viable counts of *L. bulgaricus* in synthetic media (PARADA *et al.*, 1998) and in milk (VARGA *et al.*, 1999a; de CAIRE *et al.*, 2000).

Although the data on the viable cell counts of *L. bulgaricus* showed some fluctuations, it is certain that the counts decreased in yogurts A and B but increased significantly ($p<0.05$) in yogurts C and D at the end of the storage period when compared to the beginning. The mean storage counts were below 6 log cycle in yogurts A and B, but were above 6 log cycle in yogurts C and D. The higher counts of *L. bulgaricus* in probiotic yogurts could be the result of the competition between *B. animalis* and *L. bulgaricus*. DONKOR *et al.* (2006) reported that the presence of probiotic organisms including bifidobacteria increased the proteolytic activity and improved the survival of *L. bulgaricus* in yogurt.

B. animalis cell counts were not significantly influenced by the addition of *S. platensis* in yogurt D. The viability of *B. animalis* remained well above the recommended limit of 6 log cfu g^{-1} in yogurts during the refrigerated storage. Similar findings were reported in other studies (SHAH *et al.*, 1995; DAVE and SHAH, 1997; SHIN *et al.*, 2000). In yogurt D, the viable cell counts of *B. animalis* were stable during the 28 d storage period, whereas a significant decrease was observed in the *B. animalis* counts in yogurt C at the end of the storage when compared to the first day.

In yoghurt C the viable cell counts of *B. animalis* decreased significantly ($p<0.05$) on the 7th and 28th days of storage. This could be explained by the significant increase in the *L. bulgaricus* cell counts on days 7 and 28. It has been claimed that higher counts of *L. bulgaricus* have an adverse effect on the viability of probiotic organisms because of a decrease in the pH value (SHAH *et al.*, 1995).

S. platensis biomass showed no influence on the growth of *B. bifidum* when the milk was inoculated with the mixed culture of *S. thermophilus* and *B. bifidum* (VARGA *et al.*, 1999a). On the other hand, in contrast to the results of this

study, the *Spirulina*-supplemented fermented ABT milk contained significantly ($p<0.05$) higher levels of viable bifidobacteria throughout the whole storage compared to the control product (VARGA *et al.*, 2002). This could have been due to the different starter culture used and competition among the bacteria. It can be seen that the suppressive, competitive impact of *L. bulgaricus* on *B. bifidum* was much more pronounced than the beneficial effect of adding algal biomass to yogurt containing *B. bifidum*.

CONCLUSIONS

This study demonstrated that the addition of *S. platensis* biomass increased the protein level in yogurts, and significantly ($p<0.05$) enhanced the viability of *L. bulgaricus*.

While the addition of *S. platensis* did not significantly influence the survival of *B. animalis* in probiotic yogurt, it decreased the survival of *S. thermophilus* in both traditional and probiotic yogurts. Probiotic algal yogurt has a one month shelf-life with a viable *Bifidobacterium* cell count of at least 6 log cfu g^{-1}.

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SHORT COMMUNICATION

CATTLE BREED TRACEABILITY IN MEAT AND CHEESE USING DNA POLYMORPHISMS DETECTED BY THE SNAPSHOT METHOD

TRACCIABILITÀ DELLA CARNE E DEL FORMAGGIO DI RAZZE BOVINE ATTRAVERSO L'IMPIEGO DI POLIMORFISMI DEL DNA CON LA TECNICA SNAPSHOT

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ABSTRACT

Breed identification of animal products is a fundamental tool used to control food quality, add value to regional products and exploit local breeds as well as prevent food adulteration. *Myostatin* (*MSTN*) and *Melanocortin 1 Receptor* (*MC1R*) are ideal candidate genes for molecular breed traceability of cattle because of their breed-specific variability. A novel method based on primer extension (SNaPshot) was success-

RIASSUNTO

L'identificazione della razza con cui è stato ottenuto un particolare prodotto di origine animale è un punto fondamentale nel controllo della qualità e nella valorizzazione dei prodotti ottenuti impiegando razze locali. I geni della Miostatina (*MSTN*) ed il Recettore 1 della Melanocortina (*MC1R*) sono due importanti geni candidati per la tracciabilità molecolare nella specie bovina data la loro variabilità razza-specifica. Un

- Key words: cattle, *MC1R, Myostatin,* SnaPshot, traceability -

fully used for the *MSTN* and *MC1R* genotyping of eight bovine breeds. A total of 114 samples from hair bulb and cheese were analyzed. The results obtained allowed the breeds to be identified and results were confirmed by PCR-RFLP and DNA sequencing. The proposed SNaPshot method provides rapid throughput analysis from highly fragmented DNA of processed foods from animals, such as cheese.

metodo basato sulla minisequenza del DNA, lo "SNaPshot", è stato impiegato con successo nella genotipizzazione di otto razze bovine ai loci *MSTN* e *MC1R*. Sono stati analizzati complessivamente 114 campioni di DNA ottenuti da bulbo pilifero e da formaggio. I risultati ottenuti in questo lavoro garantiscono l'identificazione della razza di provenienza applicando il metodo SNaPshot e sono stati confermati grazie ai metodi PCR-RFLP e sequenziamento. Il nuovo metodo di SNaPshot si propone come tecnica innovativa che consente di realizzare le analisi velocemente e su un gran numero di campioni anche partendo da DNA notevolmente frammentato perché isolato da matrici alimentari quali il formaggio.

INTRODUCTION

Breed certification has been recently regulated by the European Community (EC n. 1760/2000); a compulsory system of meat labelling has been established in order to check its origin. For this reason, cattle traceability has become an important priority in animal biotechnology research (POPPING, 2002). Product traceability is a fundamental tool used to control food quality, give added value to regional products and prevent food adulteration by exploiting local breeds (MCKEAN, 2001). For this reason, valid and reliable methods are necessary to detect the breed of origin of products such as milk, cheese and meat. Several studies have demonstrated the feasibility of using DNA analysis to successfully trace the origin of meat or milk to an individual animal and to infer the breed. Breed identification can be achieved either by a probabilistic approach based on hypervariable loci analysis (CIAMPOLINI *et al.*, 2006; NEGRINI *et al.*, 2007) or by using a deterministic approach supported by breed-specific allele analysis (RUSSO *et al.*, 2007; CHUNG *et al.*, 2000). Among the loci identified on livestock genomes, the *Myostatin* (*MSTN*) and *Melanocortin 1 Receptor* (*MC1R*) genes were found to be suitable for breed traceability of cattle because of their breed-specific variability.

MSTN is a member of the transforming growth factor-β superfamily and a negative regulator of skeletal muscle growth. Mutations in the *MSTN* gene are responsible for double muscling or muscular hypertrophy phenotype in many European cattle breeds (GROBET *et al.*, 1997; KAMBADUR *et al.*, 1997; MCPHERRON and LEE, 1997; CAPPUCCIO *et al.*, 1998). The *MSTN* gene has been widely studied and has a surprisingly high number of breed-specific polymorphisms (GROBET *et al.*, 1998). Since it was first

detected, the double muscling condition has become widespread among the double-muscled Piemontese cattle, leading to higher development of valuable components of the carcass.

Conversely, the *MC1R* gene, is involved in the regulation of eu-/pheo-melanin synthesis; to date, four distinct allelic forms have been described. Three *extension* alleles have been identified (KLUNGLAND *et al.*, 1995): wild-type E^+ is 954 bp long and produces a variety of colours depending on the agouti locus; the dominant E^D allele is characterized by a T > C missense mutation responsible for the black coat colour, whereas the recessive *e* allele caused by a single nucleotide deletion produces a premature stop codon that results in a red coat. The fourth allelic variant, named E^1, was firstly described by ROUZAUD *et al.* (2000) and displays a duplication of 12 nucleotides in position 669 resulting in attenuated coat colour. Table 1 reports the *MC1R* alleles and genotypes that determine the different coat colours and their distribution among cattle breeds.

Both the *MSTN* and *MC1R* genes have been widely employed as genetic markers for genotyping (FAHRENKRUG *et al.*, 1999; KARIM *et al.*, 2000; ROUZAUD *et al.*, 2000; BERRY *et al.*, 2002; MIRANDA *et al.*, 2002; CREPALDI *et al.*, 2003; DI STASIO and ROLANDO, 2005) and breed traceability (MAUDET *et al.*, 2000; ROLANDO and DI STASIO, 2006) since they display alternative allele distribution in several breeds (ROLANDO and DI STASIO, 2006; RUSSO *et al.*, 2007). However, the techniques utilized so far are neither economical, suitable or reliable for high throughput analysis, nor are they easy to perform when using fragmented DNA isolated from processed animal foods such as cheese. The aim of the current investigation is to establish and discuss a new method, using the SNaPshot technique that is useful for tracing products of specific cattle breeds (cheese) and suitable for high throughput genotyping.

MATERIALS AND METHODS

A total of 114 DNA samples were analyzed, 106 of which were isolated from hair bulb: 18 Holstein Friesian, 11 Maremmana, 27 Italian Brown, 15 Romagnola, 10 Chianina, 10 Piemontese, 10 Italian Simmental and 5 Marchigiana; 8 DNA samples were isolated from 4 different samples of Parmesan cheese. Cheese samples were provided by the DISOLOBRUNA Consortium (Bussolengo, VR, Italy).

Table 1 - Polymorphisms and their haplotype combinations in determining *MC1R* alleles and genotype fixation in different breeds.

Allele	C/T SNP in *MC1R* locus sequence position 296 (U39469)	G deletion at sequence position 310 (U39469)	Genotype	Breed
E^D	C	G	E^D/E^D or E^D/e	Holstein Friesian
E^+ or E^1	T	G	E^+/E^+ or E^+/e E^1/E^+ or E^1/E^1	Italian Brown, Chianina, Marchigiana, Maremmana, Piemontese, Podolica, Gasconne, Romagnola
e	T	(delG)	*e*/*e*	Blonde D'Aquitane, Charolaise, Limousine, Italian Simmental, Salers, Pinzgauer

DNA extraction from cheese

DNA was extracted using two different kits: Qiagen Dneasy™ Tissue Kit (Quiagen Inc, Valencia, CA, USA) and Invitrogen PureLink™ Genomic DNA Mini Kit (Carlsbad, CA, USA) according to the manufacturer's instructions. Both methods produced good quality DNA.

DNA extraction from hair bulb

Three individual hair samples were cut at 0.5 cm from the hair bulb and incubated in a PCR thermocycler at 60°C for 45 min and at 95°C for 15 min with 100 µL of Lysis Buffer (0.5% Tween 20; 0.6% NP 40, Amresco Solon, OH, USA) supplemented with 10 µL of PCR Buffer 10X, 0.85 mM of $MgCl_2$ and 0.10 mg/mL Proteinase K. DNA was then precipitated with cold 2-propanol, centrifuged for 20 min at 15,300 *g*, air dried and then used for PCR amplification.

Myostatin analysis

MSTN gene polymorphism was analyzed in 5 samples for each breed using the SNaPshot method as described below.

Polymerase chain reaction

PCR was performed to amplify a 351 bp fragment, (Genbank accession n. AB076403) in a total volume of 10 µL containing 20 ng of DNA, 1X PCR Buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.625 U Taq DNA polymerase (Promega Madison, WI, USA), and 0.3 µM of each primer (P2F-My 5' TTG GGC TTG ATT GTG ATG AA 3'and P2R-My 5' GGA AGC TAT GAA CCA AAT ATA GAC C; designed on the AB076403 sequence) under the following conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 1 min and a final extension at 72°C for 7 min. PCR products were resolved in 2% agarose gel electrophoresis in 0.5X TBE buffer, stained with ethidium bromide.

Single nucleotide primer extension (SNaPshot)

Amplification products were purified from primers and dNTP residues through Exo-SAP treatment. A total volume of 10 µL containing 5 µL of PCR product, 5 U of *Exo*I (New England BioLabs, Hertfordshire, UK), 1X Buffer Exo and 1U SAP (Shrimp Alkaline Phosphatase, Promega, Madison, WI, USA) was incubated for 1 hour at 37°C and successively treated for 15 min at 75°C to inactivate the enzyme.

The SNaPshot reaction was performed according to manufacturer's instructions, with minor modifications, in a 5.5 µL total volume containing 1.0 µL of purified PCR, 1.25 µL SNaPshot ready reaction premix (Applied Biosystems, Foster City, CA, USA), 1.35 µM extension primer (Sn-MyF 5' CCA ATT ACT GCT CTG GAG AAT 3' and Sn-MyR 5' CTT TTG CAA AAA TAC AAA TTC A 3'; designed on the AB076403 sequence). The SNaPshot reaction mix amplification condition was: 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 30 s. Upon completion, 0.05 U SAP was added and the reaction mixture was again incubated for 60 min at 37°C and 15 min at 75°C. Before loading onto the ABI Prism 310 Sequencer, 9 µL Hi-Dye formamide and 0.3 µL LIZ 120 internal standard (Applied Biosystems, Foster City, CA, USA) were added to 0.5 µL of reaction mixture and samples were heated to 95°C for 5 min. The fragments were analyzed using ABI Prism GeneMapper® version 4.0 (Applied Biosystems, Foster City, CA, USA).

MC1R analysis

The *MC1R* polymorphisms were analyzed in all samples through PCR-RFLP (restriction fragment length polymorphism) and also in 5 samples for each breed using the SNaPshot technique. In the Italian Simmental breed, all 10 samples were analyzed with both techniques.

PCR-RFLP analysis

A single PCR was carried out to amplify a 138 bp fragment, (Genbank accession n. U39469). PCR reactions were performed in a total volume of 30 μL containing 20 ng of genomic DNA, 1X PCR Buffer, 2.0 mM $MgCl_2$, 200 μM dNTPs, 1 U Taq DNA polymerase (Promega Madison WI, USA), 0.15 μM of each primer (MC1RF 5' CAA GAA CCG CAA CCT GCA CT 3'and MC1RE6 5' GCC TGG GTG GCC AGG ACA 3'; designed by KLUNGLAND *et al.*, 1995) under the following conditions: initial denaturation at 94°C for 3 min, followed by 40 cycles at 94°C for 30 s, 63°C for 45 s, 72°C for 1 min, and a final extension at 72°C for 5 min. PCR products were resolved in 2% agarose gel electrophoresis in 0.5X TBE buffer, stained with ethidium bromide. PCR products were double digested in two separate reactions, with *Msp*I and *Aci*I enzymes, according to manufacturer's instructions (New England Biolabs, Beverly, MA, USA). Fragments were resolved on a 4% agarose gel and visualized on a UV light source after staining with ethidium bromide.

Single nucleotide primer extension (SNaPshot)

The assay was carried out as previously described using the following SNaPshot primers: Sn-MC1R_CT-F 5' CGT GCT GGA GAC GGC AGT CAT GC 3' and Sn-MC1R_DelGR2 5' $(C)_{30}$CGC CTG GGT GGC CAG GAC AC 3', designed on the U39469 sequence.

RESULTS AND DISCUSSION

Myostatin gene analysis by means of the SNaPshot method using both forward and reverse primers revealed the A/G polymorphism. The Piemontese breed presented the A nucleotide at position 4,942 of the AB076403 sequence, whereas the other breeds revealed the G nucleotide at the same position. Electropherograms were obtained using the reverse extension primer, thus the alleles are T and C (Fig. 1). The other breeds revealed a C nucleotide at the same position. All samples were assigned correctly, but in the Romagnola and Piemontese breeds, one animal among the five analyzed for each breed was heterozygous (A/G). The forward and reverse sequence in both samples confirmed this result.

The *MC1R* gene was genotyped for polymorphism C/T (E^D allele), and the deletion of G (*e* allele) was successful using the same SNaPshot reaction mix containing both the extension primers to analyze two polymorphic positions in just one reaction. All the genotypes detected were in agreement with the allele distribution in the breeds. Fig. 2 shows the two peaks obtained for the E^D allele (Holstein Friesian; panel A), the peaks due to E^+ or E^1 alleles (Maremmana, Italian Brown, Romagnola, Chianina, Piemontese and Marchigiana; panel B) and the *e* allele (panel C). Five of the 10 samples of the Italian Simmental breed were heterozygous for the *e* allele (Fig. 2, panel D), confirming that the *e* allele is not fixed (RUSSO *et al.*, 2007).

The *MC1R* polymorphism detection SNaPshot method has been successfully applied to discriminate among genotypes on DNA isolated from hair bulb, as well as on DNA isolated from cheese. Moreover, genotyping by means of PCR-RFLP was performed to compare the results; both methods correlated perfectly (Photo 1). However, it should be noted that the restriction analysis conducted on the 138 bp fragment required more experience because the shorter fragments were difficult to detect. In contrast, the reading of the SNaPshot peak could be easily carried out. Other PCR-RFLP methods based on longer fragments were tested (data not shown); however, they were not suitable for amplifying a degraded DNA template such as the DNA isolated from cheese.

Three alleles are not sufficient for

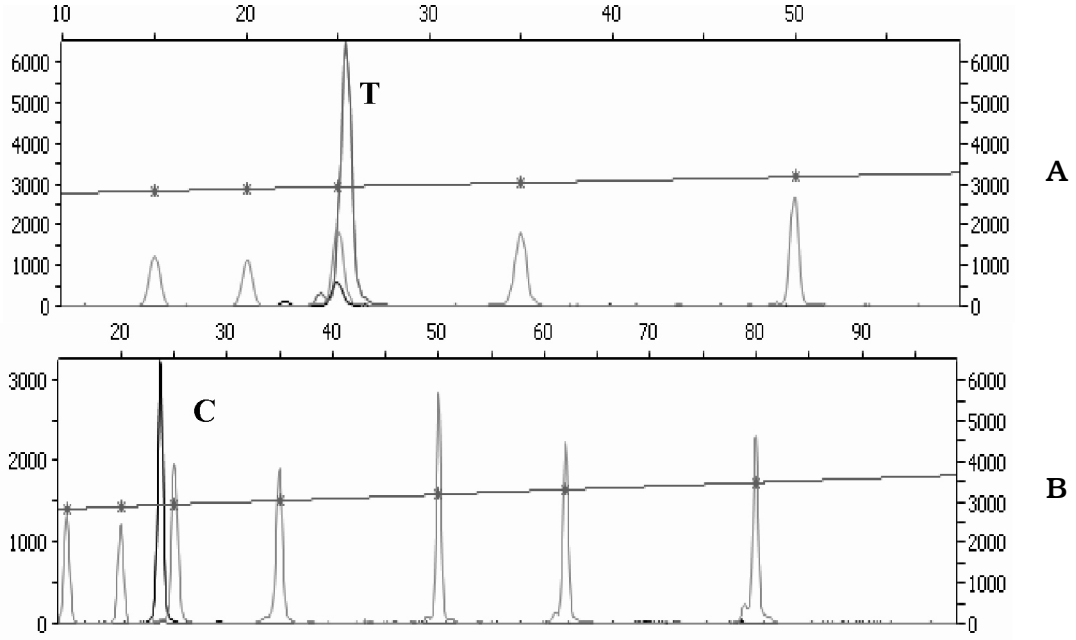


Fig. 1 - Example of an electropherogram resulting from *myostatin* (*MSTN*) genotyping using the SNaPshot method.
Panel (A), the T allele of Piemontese cattle; panel (B), the C wild-type allele. The products were obtained using the reverse 22-bp–long SNaPShot primer. Orange peaks represent the standard LIZ 120 fragments. The y-axis reports the height of the peaks and size of the fragments expressed in base-pairs.

breed assignment, but this statement is true only when the SNPs are used as anonymous markers in the probabilistic approach. The scenario changes completely when SNPs are in codifying gene regions and modify phenotypic traits under selection pressure (e.g., coat colour). In fact, since the time of the introduction of the first modern cattle breeds, coat colour has usually been considered an aid to identifying breeds. Therefore, registration of the animals to herd-books requires coat colour specificity. These rules set out in establishing distinctive coat colours in the vast majority of European cattle breeds, including those that were considered in our study (RUSSO *et al.*, 2007). By adopting a deterministic approach, statistical analysis was not required, since SNPs are fixed and generate different fixed haplotypes which allow breed classification. For example, the E^D allele in the *MC1R* locus was detected only in black breeds (ROUZAUD *et al.*, 2000; CREPALDI *et al.*, 2003; ROLANDO and DI STASIO, 2006).

The present study demonstrates that the SNaPshot technique can be reliably employed to analyze *MC1R* and *Myostatin* genotypes. Even if other methods based on PCR-RFLP have been designed to detect this polymorphism (DI STASIO and ROLANDO, 2005), the novel SNaPshot method offers improvements. Single nucleotide primer extension techniques are reliable and fast and the purification steps and extension reaction could be easily automated which allows rapid, simple and high throughput analysis. In addition, the SNaPshot method can be employed to analyze numerous DNA samples isolated from animal products (e.g., cheese and meat) (REALE *et al.*, 2006). SNaPshot was suc-

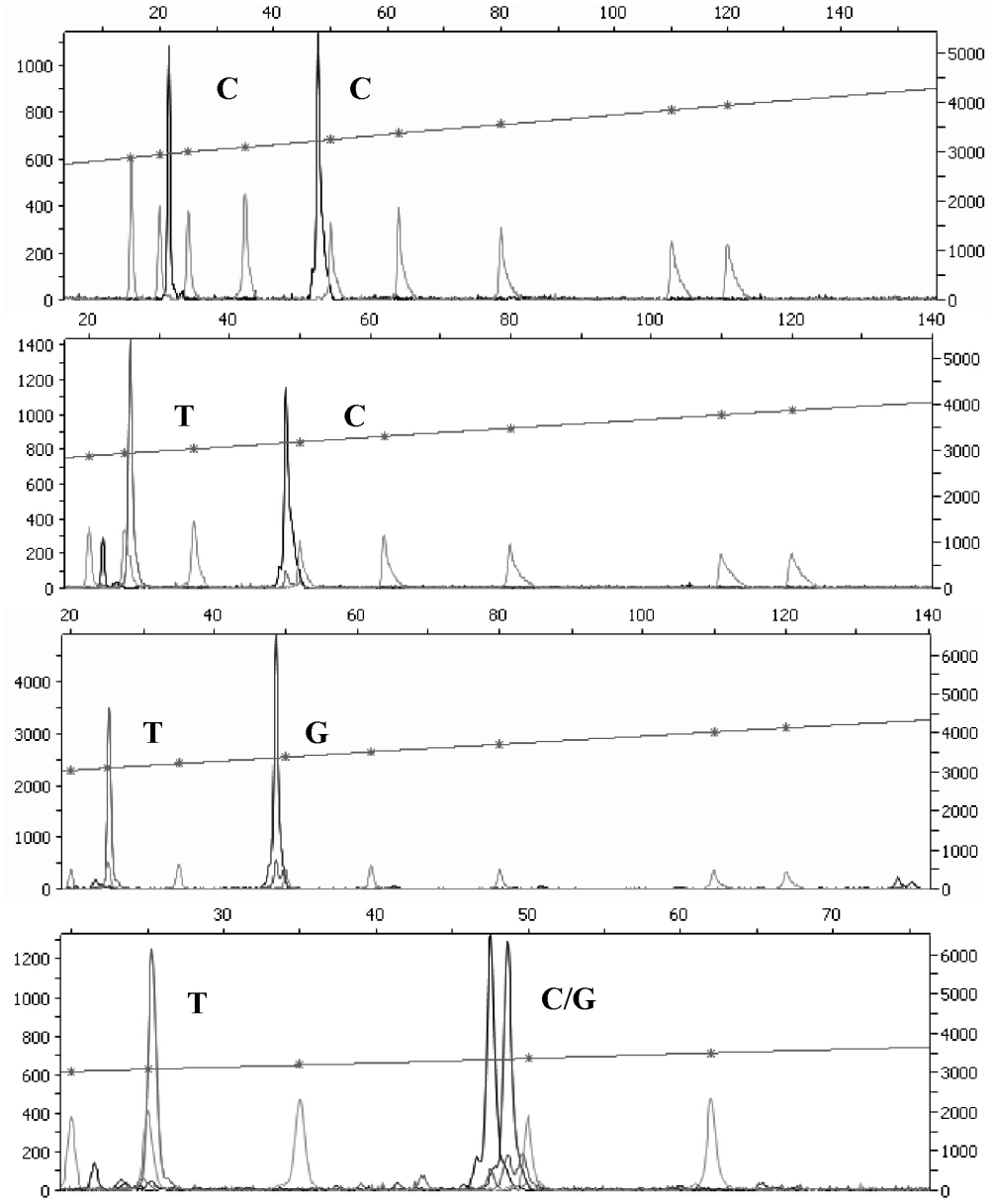


Fig. 2 - Example of an electropherogram resulting from *Melanocortin-1 Receptor* (*MC1R*) genotyping using the SNaPshot method.
Panel (A), result obtained analyzing an animal E^D/E^D (genotype CC/CC) at the *MC1R* locus. Panel (B), the E^+/E^+ or E^1/E^+ or E^1/E^1 (genotype TC/TC).
Panel (C), the *e*/*e* animal (genotype TG/TG).
Panel (D), an Italian Simmental heterozygous (having a T nucleotide at a homozygous state in the first position and a C/G in the second position).
The products were obtained using a 23-bp–long forward SNaPShot primer to genotype the first position, and a reverse 50-bp–long SNaPShot primer to genotype the second polymorphic position. Orange peaks are the standard LIZ 120 fragments. The y-axis reports the height of the peaks and size of the fragments expressed in base-pairs.

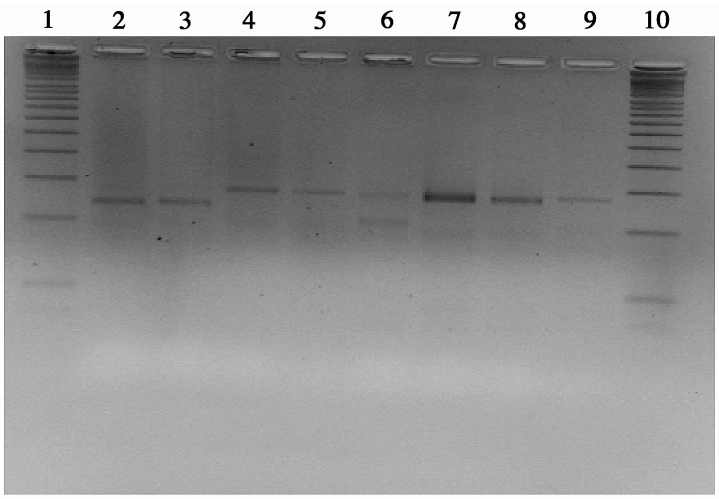


Photo 1 - Example of *Melanocortin-1 Receptor* genotyping using *Msp*I and *Aci*I PCR-RFLP methods.

Lane 1: 50 bp ladder (Sigma Aldrich Missouri, USA)
Lane 2: *Msp*I digestion; genotype G/G in an E^D/E^D animal (118bp+20bp)
Lane 3: *Msp*I digestion; genotype G/G in an E^+/E^+ or E^1/E^+ or E^1/E^1 animal (118bp+20bp)
Lane 4: *Msp*I digestion; genotype delG/delG in an e/e animal (138bp)
Lane 5: 138 bp amplified fragment (138 bp)
Lane 6: *Aci*I digestion, genotype C/C in an E^D/E^D animal (97bp+33bp+8bp partially indigested)
Lane 7: *Aci*I digestion, genotype T/T in an E^+/E^+ or E^1/E^+ or E^1/E^1 animal (130bp+8)
Lane 8: *Aci*I digestion, genotype T/T in an e/e animal (130bp+8)
Lane 9: 138 bp amplified fragment (138bp)
Lane 10: 50 bp ladder (Sigma Aldrich Missouri, USA)
Please note that shorter fragments are not visible on agarose gel.

cessfully used for the characterization and genotyping of the caprine κ-casein variants (YAHYAOUI *et al.*, 2003). Moreover, because DNA samples isolated from processed foods are heavily fragmented, their large fragment size that could be easily evaluated on agarose gel after RFLP analysis cannot be easily obtained. On the contrary, the SNaPshot method can also be performed on very short fragments containing the polymorphic point.

Genotyping of the *MC1R* locus can be applied to detect Holstein Friesian milk contamination or the addition of it in Italian Brown cheese labelled as a monobreed product. Moreover, it can be useful to discriminate between meat with the Protected Geographical Indication (Chianina, Marchigiana, Romagnola) and the main imported French breeds (Limousine and Charolaise). *Myostatin* locus genotyping is useful in the Piemontese Consortium labelled meat traceability, since the A allele appears only in this breed. The *MSTN* and *MC1R* genotyping method tested in this study on eight Italian bovine breeds and cheese samples can be employed in mono-breed–labelled product identification and as a deterrent against fraud.

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SHORT COMMUNICATION

ALLERGIC RESPONSE TO A PARA-AMINO COMPOUND

RISPOSTA ALLERGICA AI COMPOSTI PARA-AMINO

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ABSTRACT

A 45-year-old woman reported recurrent episodes of urticaria, which had first appeared about one year earlier, when she started a diet which included eating frozen vegetable soup almost daily. Patch tests with the standard European series, food preservatives and colourings were performed and the results were only positive (+++, following the International Contact Dermatitis Research Group criteria) for *p*-aminoazobenzene. After avoiding eating

RIASSUNTO

Una donna di 45 anni riferiva episodi ricorrenti di orticaria, che erano esorditi circa un anno prima. Veniva eseguito patch test per la serie Standard europea, conservanti alimentari e coloranti, con risultato positivo (+++, seguendo i criteri dell'International Contact Dermatitis Research Group) solo per il *p*-aminoazobenzene. La paziente riferiva che questi episodi avevano avuto inizio 1 anno prima quando la stessa aveva iniziato una dieta

- Key words: azo dyes, chronic urticaria, *p*-aminoazobenzene, para-amino compounds, systemic contact dermatitis -

frozen vegetable soup from a coloured plastic wrapper, which was probably coloured with para-amino compounds that cross-reacted with *p*-aminoazobenzene, the patient's urticaria rapidly disappeared.

mangiando frequentemente minestrone surgelato. Dopo aver evitato l'ingestione di minestrone surgelato in buste colorate, che probabilmente contenevano composti para-amino cross-reagenti con il *p*-aminoazobenzene, la paziente presentava una rapida scomparsa dell'orticaria.

INTRODUCTION

p-Aminoazobenzene (Fig. 1) is an azo dye which belongs to a broad group of chemicals that are used in a variety of applications, such as in hair dyes, food, and in the paper and textile industries (SEIDENARI *et al.*, 1997; DEVOS and VAN DER VALK, 2001).

Contact allergies to azo dyes are quite frequent (0.05-15.9%) and some authors have noted that the incidence is rising (UTER *et al.*, 2001; PRATT and TARASKA, 2000). To date systemic contact dermatitis (SCD) to *p*-aminoazobenzene has not been reported.

CASE REPORT

A 45-year-old woman reported recurrent episodes of systemic urticaria which had first appeared about one year earlier. Treatment with antihistamines and corticosteroids only gave temporary relief. The patient was advised to follow an elimination diet and after 20 days the urticaria disappeared. Adverse drug reactions were excluded and all the diagnostic procedures for screening the possible causes of urticaria were carried out.

MATERIALS AND METHODS

Results from differential blood count, erythrocyte sedimentation rate (ESR), Anti Nuclear Antibodies (ANA), thyroid hormones (FT3, FT4, TSH) and autoantibodies (TPOAb, TMAb), autologous serum skin test, *Helicobacter pylori* breath test and stool examination for worm eggs and/or parasites were negative.

Therefore allergological evaluations were carried out including a Skin Prick Test with commercial extracts (Stallergenes, Saronno, Varese, Italy) of the most common inhalants (house dust mites, moulds, *Parietaria judaica*, grass pollen, dog and cat dander) and food (milk proteins, egg yolk, egg white, cod, shrimp, *Anisakis simplex*, peanut, soybean, tomato, wheat flour, celery, carrot, potato, bean, eggplant, apple, orange). A patch test was conducted with commercial series: standard European series, food preservatives and colourings (Lofarma, Milan, Italy); specific IgE antibodies (Phadia CAP System fluorimetric test, Uppsala, Sweden) for the above reported inhalants and food allergens and a Double Blind Placebo Controlled Food Challenge (DBPCFC), the gold standard test in food allergy (BOCK *et al.*, 1988).

RESULTS AND DISCUSSION

The skin prick tests for the common inhalants and foods were negative as were the determinations of specific IgE (<0.35 KU/L) for the same allergens.

The patch test was strongly positive

after 72 h (vesicles spreading over the chamber mark; +++ following the International Contact Dermatitis Research Group criteria) for *p*-aminoazobenzene only. A second reading after 96 h confirmed positivity.

Further investigation of the patient revealed that the urticaria had started a year earlier when she had begun a diet which included eating frozen vegetable soup nearly every day.

A patch test with a 1 cm^2 piece of the coloured plastic wrapping, which contained the frozen vegetable soup, was applied to the patient's back with Scanpor tape; the test was evaluated after 72 h and the results were positive (vesicles; ++ following the International Contact Dermatitis Research Group criteria). A second reading after 96 h confirmed the positivity.

A Double Blind Placebo Controlled Food Challenge (DBPCFC) was performed first with a vegetable soup from a transparent plastic wrapping and the results were negative. A week later, the test was carried out with the kind of vegetable soup that the patient usually consumed that was packaged in a coloured plastic wrapper. The systemic urticaria appeared within two h.

The coloured plastic wrapping, which is in direct contact with the vegetable soup was considered to be the cause of the SCD.

Reactive dyes consist of a direct colorant, with an azo or, more rarely, an anthraquinone structure or a phthalocyanin derivative group, which is connected to a reactive group, that is capable of bonding to amine or sulfhydryl groups of proteins, by covalent bonds. These could be responsible for the sensitizing process (BURKINSHAW, 1991). Allergic sensitivity to different chemicals with a para di-substituted structure (MAYER, 1928), often termed para-amino compounds, is frequent (BRUZE, 1984). The degree of cross-reactivity between members of this chemical group is quite variable, given that the formation of reactive nucleophilic quinine imines is the presumed mechanism that leads to common haptens (UTER *et al.*, 2002).

p-Aminoazobenzene appears to be the most suitable screening allergen for para-amino compound allergies and shows cross-reactivity with several molecules including *p*-phenylenediamine, Disperse Orange 3 and *p*-toluylenediamine, *p*-aminophenol and 4,4'- diaminophenylmethane (UTER *et al.*, 2002).

Migration is a property common to many colorants used in food packaging materials, that is; they are transferred to foodstuffs even though the packaging materials and articles should not transfer their constituents to the foodstuffs in quantities that could endanger human health (ARVANITOYANNIS and BOSNEA, 2004). The allergic response (SCD), observed in this patient could have been due to a para-amino compound (probably an oxidative or azoic dye) present in the coloured plastic, soup wrapper that cross-reacted with *p*-aminoazobenzene. After the diagnosis, the patient remembered a previous erythematous reaction to a pair of nylon tights; *p*-aminoazobenzene can also be found in nylon hosiery (SEIDENARI *et al.*, 2002).

SCD, in fact, develops after oral or parenteral exposure to an allergen in a topically sensitized individual. This is believed to be the results of the hematogenous spread of the allergen, such as antibiotics, penicillin or metals and flavouring agents that provoke a cutaneous reaction. It can be manifested as dyshidrotic eczema, a flare of patch test reaction sites, generalized maculopapular eruption, erythroderma, or less commonly as multiform erythema, urticaria and vasculitis. Systemic effects such as rhinitis, conjunctivitis, headache, gas-

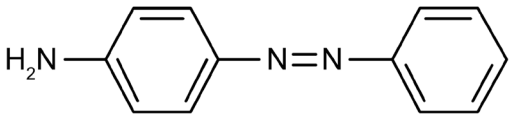


Fig. 1 - Chemical structure of *p*-aminoazobenzene.

trointestinal complaints, or anaphylaxis may also occur. Closed patch testing to potential allergens for SCD is considered a reliable and safe means of testing (BRANCACCIO and ALVAREZ, 2004).

It has been shown in nickel-sensitised patients with allergic contact dermatitis syndrome that a mixed cytokine pattern is present as peripheral blood mononuclear cells from nickel-allergic individuals responded to nickel with significantly higher IL-4, IL-5, IL-13 (Th-2 cytokines) and IFN-γ (Th-1 cytokine) production to non-allergic controls (JAKOBSON *et al.*, 2002).

Of course the hypothetical explanation of this case is merely speculative, since a simultaneous occurrence of positive patch tests to different allergens cannot be automatically equated with allergenic cross-reactivity and previous exposure to other compounds could be the cause of the co-sensitization. For example, a cross-reaction of *p*-aminoazobenzene with sulphonamide cotrimoxazole may occasionally occur (DE WAARD-VAN DER SPEK and ORANJE, 2008).

In conclusion the data suggest that para-amino dyes may be released from coloured plastic packaging and be transferred to the foodstuff in quantities that are sufficient to trigger allergic reactions in sensitive subjects.

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