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Activity of selected essential oils on spoiling fungi cultured from Marzolino cheese

Simona Nardoni[1,A-F], Carlo D'Ascenzi[1,A,F], Irene Caracciolo[1,B,F], Gaia Mannaioni[1,B,F], Roberto Amerigo Papini[1,F], Luisa Pistelli[2,B,F], Basma Najar[2,B,F], Francesca Mancianti[1,A,C-F]

[1] *Università di Pisa, Dipartimento di Scienze Veterinarie, Pisa, Italy*
[2] *Università di Pisa, Dipartimento di Farmacia, Pisa, Italy*
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Abstract

Microscopic fungi can be present on a variety of foodstuff, including cheese. They can be responsible for fungal spoilage, causing sensory changes making food unacceptable for human consumption, and posing severe health concerns. Furthermore, some of these organisms are able to resist antimicrobial preservatives provided for by law. Antifungal activity of 15 chemically defined EOs, alone and in mixture, were checked by a microdilution test against isolates of Penicillium funiculosum and *Mucor racemosus* cultured from rinds of Marzolino, a typical Italian fresh pecorino cheese. *Origanum vulgare* yielded the lowest MIC values, followed by *Salvia sclarea*, *Ocimum basilicum* and *Cymbopogon citratus*, while *Citrus paradisi* and *Citrus limon* were not active. All mixtures showed antifungal activity at lower concentration with respect to MIC values of each EO component, when not in combination. This study is the first to describe the setting up of EOs mixtures to limit spoiling moulds.

Key words

essential oils, mixtures, cheese, spoiling fungi, Penicillium funiculosum, Mucor racemosus

INTRODUCTION

Italian cheese is one of the most appreciated symbols of the 'made in Italy' food products worldwide. This rich Italian tradition includes 52 PDOs/PGIs products (Protected Designation of Origin/Protected Geographical Information), over 237 registered in Europe, only in second place after France with 55 cheeses, and 492 further products registered as traditional cheeses by the Italian Ministry of Agriculture [1]. The cultural and economic value of this huge gastronomic resource is based on the quality of the raw materials used, and the need for excellence at all stages in the processing, interpreting the traditional methods, without additives or other chemicals.

Microscopic fungi such as moulds and yeasts are ubiquitous organisms, colonizing a broad variety of foodstuffs, including cheese. A wide diversity of fungal species can be present on these products, depending on the microbiota of the milk, on the handling processes and by organisms occurring in the productive and storage environments.

Most of these cheeses, in fact, are obtained through an ageing process that can be short, medium or long, in order to develop their unique attributes. During this period, spoilage by ubiquitous moulds can occur, representing a critical point in the production process. Moulds are important spoilage agents in cheese [2], affecting both colour and texture. These organisms can tolerate refrigeration temperatures and low levels of water activity, are able to grow on cheese rind, and some of them resist antimicrobial preservatives provided for by law, such as sorbate (Reg. 1333/2008).

Address for correspondence: Simona Nardoni, Università di Pisa – Dipartimento di Scienze Veterinarie, viale delle Piagge, 56124 Pisa, Italy
E-mail: simona.nardoni@unipi.it

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Essential oils (EOs) are mixtures of aromatic oily liquids extracted from different parts of plants, usually by steam distillation [3]. Several of them are characterized by antimicrobial activities, mainly due to their content in terpenes and phenylpropanoids [4, 5], and have been used both in therapy [6] and to control spoilage flora of foodstuff [7]. Natural plant materials have been used as food preservatives against both bacteria and fungi [8, 9]. Consequently, natural mixtures have been added to a variety of foodstuff, including cheese, to control fungi producing visible fungal growth, off-flavours and/or mycotoxins [2]. Few data are available about the application of EOs for their antifungal activity in food such as cheese. To the best of the authors' knowledge, only two papers refer to the use of such compounds as mould inhibitors in the product [10, 11].

For these reasons, the aim of the presented study is to assess the antimycotic activity of some EOs against moulds spoiling Marzolino, a fresh, typical pecorino cheese from Tuscany, Italy.

MATERIALS AND METHOD

Fungal species employed in the present study were isolated from the rinds of Marzolino cheese showing an overgrowth of spoiling fungi occurring during ripening process. Fungal growth started from the first days post-ripening and increased until the end of the cheese maturation period (day 20), making the product unsuitable for sale.

Moulds were obtained as described by Samson et al. [12], with slight modification. Briefly, a 1cm^2 area of mouldy rind was collected by scraping with a sterile blade. Samples were dissolved in peptone water and homogenized using a Stomacher 80 (Pbi International, Milan, Italy). To achieve

identifiable fungal colonies, a dilution plating technique was employed starting from 10^{-1} to 10^{-6}, then 0.1 ml of each suspension was seeded onto Potato Dextrose Agar (PDA). Plates were incubated at 25 °C until a noticeable fungal growth was observed. Identification of fungal isolates was achieved by macro- and microscopic morphology. Results of morphological identification were confirmed by PCR [13, 14] and, when required, by sequencing.

EOs of *Origanum vulgare, Ocimum basilicum, Foeniculum vulgare, Thymus vulgaris, Illicium verum, Syzygium aromaticum, Salvia sclarea, Origanum majorana, Rosmarinus officinalis, Citrus aurantium, Citrus paradisi, Citrus limon, Citrus sinensis, Citrus bergamia, Cymbopogon citratus* were checked for their antimycotic activity. These products were selected for their proven antimicrobial efficacy and for their traditional usage in foodstuff preservation.

All EOS were kindly provided by Flora s.r.l. (Lorenzana, Pisa, Italy) and were chemically defined as reported below.

The MIC value of selected EOs was assessed by means of a microdilution test, starting from a 5% dilution, following the method described by the Clinical and Laboratory Standards Institute $M38A_2$ (CLSI 2008) [15]. EOs showing a satisfactory antifungal activity and derived from plants traditionally employed in artisanal cheese already present on the market, were selected to prepare 3 mixtures composed as follows: *S. aromaticum, S. sclarea, O. vulgare,* 1% each (M1), *S. aromaticum, S. sclarea* and *C. citratus* 1% each (M2) and *C. citratus* and *T. vulgaris* (M3) 1.5% each. To better understand the measurement of the effect of EOs combinations in the mixtures, the FIC Index (FICI) was calculated, as reported by Rosato et al. [16]. The FICI was interpreted as: (i) a synergistic effect when ≤0.5; (ii) an additive or indifferent effect when >0.5 and ≤1 and (iii) an antagonistic effect when>1. The fungistatic or fungicidal activities of EOs mixtures were evaluated re-seeding inocula in which noticeable growth was not present, after washing with saline, onto a fresh PDA.

The Gas Chromatography – Mass Spectrometry (GC-MS) analysis were accomplished with an HP-5890 Series II instrument equipped with a HP-Wax and HP-5 capillary columns (both 30 m X 0.25 mm, 0.25 μm film thickness), working with the following temperature programme: 60 °C for 10 min, rising at 5 °C/min to 220 °C. The injector and detector temperatures were maintained at 250 °C; carrier gas, nitrogen (2 mL/min); detector, dual FID; split ratio 1:30. The volume injected was 0.5 μL. The relative proportions of the oil constituents were percentages obtained by FID peak-area normalization without the use of a response factor. GC-MS analyses were performed with a Varian CP-3800 gas chromatograph equipped with a DB-5 capillary column (30 m X 0.25; coating thickness, 0.25 μm) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions were as follows: injector and transfer line temperature, 220 and 240 °C at 3 °C, respectively; oven temperature, programmed from 60 – 240 °C at 3 °C/min; carrier gas, helium at 1 mL/min; injection, 0.2 μL (10% hexane solution); split ratio, 1:30. Identification of the constituents was based on comparison of the retention times with those of authentic samples, comparing their linear retention indices relative to the series of n-hydrocarbons, and on computer matching against commercial and home-made library mass spectra built up from pure substances and components of known oils and MS literature data [17, 18].

RESULTS

Cultures yielded *Penicillium funiculosum* and *Mucor racemosus*. Identification based on morphological features was confirmed by PCR and sequencing for both cultured fungi. The composition of EOs examined is shown in Tables 1 and 2.

The MIC values of effective EOs were higher against *P. funiculosum*, than *M. racemosus,* with the exception of *C. citratus. O. vulgare* EO presented consistent antifungal activity against both fungal species tested, followed in descending order by *S. sclarea*, *C. citratus* (against *M. racemosus*) and *O. basilicum* (against *P. funiculosum*), *T. vulgaris* and *S. aromaticum*. Other oils showed lower antigrowth effect, while *C. paradisi* and *C. limon* were not active. Detailed MIC data for both fungal species are presented in Table 3.

All mixtures showed antifungal activity at lower concentration with respect to MIC values of each EO not in combination. In particular, M2 appeared to be the most effective, providing MIC values of 0.5% and 0.25% against *P. funiculosum* and *M. racemosus*, respectively. FICI indicated that all the components of M2 had a synergistic effect for both fungi (<0.5). EOs mixtures had a fungistatic activity at the above-reported MIC values in all cases.

Regarding the chemical composition of EOs used in the present study, the most abundant components of *O. vulgare* oil were carvacrol and p-cymene. *S. sclarea* was rich in linalool acetate, while neral and geranial represented the main constituents of *C. citratus* EO. *O. basilicum* was characterized by high content of linalool and eugenol. Thymol and p-cymene were the prevailing molecules present in *T. vulgaris*, and anethol was the predominant compound recognized in *S. aromaticum*.

DISCUSSION

Fungal spoilage is a process causing sensory changes that can make foods unacceptable for human consumption, and may pose severe health concerns. The present study is the first to describe the setting up of essential oils (EOs) mixtures versus two spoiling moulds cultured from rinds of Marzolino, a typical Italian fresh pecorino cheese.

The results obtained are in agreement with the findings of Felšöciová et al. [19], reporting the activity of *O. vulgare* EO against *Penicillium* spp. *in vitro*. Those Authors reported a fungicidal activity of this EO, while in the current study the effect was fungistatic. Kazemi et al. [20] evaluated the effect of *O. vulgare* against *Mucor* sp., referring to a good antifungal activity.

The antifungal effectiveness of *S. sclarea* (MIC 1.25%) and *O. basilicum* EOs (MICs 1.25% and 2.5%) represents an interesting finding, confirming the data provided by Azizkhani et al. [21] against *Aspergillus flavus*. Conversely, Yousefzadi et al. [22] and Cenci et al. [23] did not report antifungal activity of *S. sclarea* versus *Aspergillus niger* and *Penicillium* sp., respectively. *C. citratus* showed a good antimycotic effect against Penicilli in food [10, 24], and it is reported to strongly inhibit *Rhizopus stolonifer* [25], a mould taxonomically related to *Mucor*.

Surprisingly, in this study *T. vulgaris* did not yield a marked antimycotic activity, in contrast with reports from literature [24, 26, 27].

Table 1. Chemical composition of investigated essential oils (EOs). Compounds present at less than 1.0% were not encountered

	Component	Class*	LRI	*O.v*	*O.b*	*F.v*	*T.v*	*I.v*	*S.a*	*S.s*	*O. m*	*R.o*	*C.a*	*C. p*	*C.l*	*C.s*	*C. b*	*C.c*
1	tricyclene	mh	926			8.6		1.4							1.9		1.1	
2	α-pinene	mh	940									37.9						
3	camphene	mh	953									5.4						1.1
4	sabinene	mh	976								3.2				2.3		1.1	
5	β-pinene	mh	980			1						5			11.9		5.4	
6	6-methyl-5-hepten-2-one	nt	985															1.8
7	myrcene	mh	991	2.2							1.6	1.6	1.9	2.2	1.8	2.3	1	
8	α-phellandrene	mh	1005			2.2												
9	α-terpinene	mh	1018	2.1							4.7							
10	o-cymene	mh	1026									1.1						
11	p-cymene	mh	1026	9.3		1.9	15.3				4.2							
12	limonene	mh	1031			6.5		3.9			2.1	3.3	94.7	92.2	65.7	95.5	33.2	2
13	β-phellandrene	mh	1031															
14	1,8-cineole	om	1033		5.9							22						
15	(Z) β-ocimene	mh	1040															
16	(E) β-ocimene	mh	1050															
17	γ-terpinene	mh	1062	5.3			2.9				7.9				9.3		6.4	
18	cis-sabinene hydrate	om	1068								3.2							
19	cis-linalool oxide (furanoid)	om	1074							2.2								
20	fenchone	om	1087			20.1												
21	terpinolene	mh	1088								1.5							
22	trans-linalool oxide (furanoid)	om	1088							1.8								
23	trans-sabinene idrato	om	1097	1.8			3.8				12.8							
24	linalool	om	1098		46					8.1							14.2	1.5
25	cis-rose oxide	om	1111															
26	camphor	om	1143									7.6						
27	menthone	om	1154															
28	isomenthone	om	1164															
29	borneol	om	1165				1.6					2						
30	4-terpineol	om	1177				2.4				17.6							
31	α-terpineol	om	1189								2.7							
32	unknown						1.7											
33	menthyl chavicol(=estagol)	pp	1195		1.1	8.6												
34	citronellol	om	1228															
35	thymol methyl ether	om	1232				1.7											
36	neral	om	1240															35.2
37	geraniol	om	1255								2.7							4.4
38	linalool acetate	om	1257							54.7	3.2		1.4				31.7	
39	(E) cinnamaldehyde	nt	1266															
40	geranial	om	1270												1.2			38.4
41	citronellyl formate	om	1280															
42	(E) anethol	pp	1283			46.9		89.8										
43	Isobornyl acetate	om	1285		1.6							3.3						
44	bornyl acetate	om	1285															
45	thymol	om	1290				52.6											
46	geranyl formate	om	1298															
47	carvacrol	om	1298	65.9							20.8							
48	unknown									5.6								
49	unknown									7.2								
50	α-limonene diepoxide	om	1347							8.6								

Table 1. Chemical composition of investigated essential oils (EOs) (Continuation)

	Component	Class*	LRI	*O.v*	*O.b*	*F.v*	*T.v*	*I.v*	*S.a*	*S.s*	*O. m*	*R.o*	*C.a*	*C. p*	*C.l*	*C.s*	*C. b*	*C.c*
51	eugenol	pp	1356		11.5				77.9									
52	α-copaene	sh	1376															
53	geranyl acetate	om	1383															4.2
54	β-elemene	sh	1392		2.2													
55	β-caryophyllene	sh	1418	3.7			6.8		8.9		1.7	4.1						2.3
56	trans-α-bergamotene	sh	1437		3.6													
57	aromadendrene	sh	1439															
58	α-guaiene	sh	1440		1.1													
59	α-humulene	sh	1454															
60	(E)-β-farnesene	sh	1458															
61	germacrene D	sh	1481		3.5													
62	α-muurolene	sh	1499								1.4							
	α-bulnesene	sh	1505		2													
63	trans-γ-cadinene	sh	1513		2.8													1.2
64	eugenol acetate	pp	1524						12.2									
65	δ-cadinene	sh	1524				1											
66	citronellyl butyrate	om	1532															
67	geranyl butyrate	om	1564															
68	caryophyllene oxide	os	1581							4.8								
69	(E)-2-phenyl ethyl tiglate	nt	1583															
70	1,10-di-epi-cubenol	os	1614		1													
71	T-cadinol	os	1640		5.8													
72	(Z)-citronellyl tiglate	os	1658															
73	geranyl tiglate	os	1696															
74	benzyl benzoate	nt	1762															
75	scareol	od	2223							1.3								

*mh – monoterpene hydrocarbons; om – oxygenated monoterpenes; sh – sesquiterpene hydrocarbons; os – oxygenated Sesquiterpenes; od – oxygenated diterpenes; nt – non-terpene derivatives; pp – phenylpropanoids.
LRI – Linear Retention Index.
O.v. – Origanum vulgare; O.b. – Ocimum basilicum; F.v. – Foeniculum vulgare; T.v. – Thymus vulgaris; I.v. – Illicium verum; S.a. – Syzygium aromaticum; S.s. – Salvia sclarea; O.m. – Origanum majorana; R.o. – Rosmarinus officinalis; C.a. – Citrus aurantium;
C.p. – Citrus paradisi; C.l. – Citrus limon; C.s. -itrus sinensis; C.b. -itrus bergamia; C.c. -ymbopogon citratus.

Table 2. Groups of chemical compounds of investigated essential oils (EOs)

	O.v	*O.b*	*F.v*	*T.v*	*I.v*	*S.a*	*S.s*	*O. m*	*R.o*	*C.a*	*C. p*	*C.l*	*C.s*	*C. b*	*C.c*
Monoterpene hydrocarbons	22.5	2.3	22.1	21.5	7.3			27.7	56.5	97.4	96.2	94.3	98.7	49	3.9
Oxygenated monoterpene	71.2	56.1	21.1	64.1	0.7		78.2	66.6	36.7	1.9	0.5	3.6	0.6	48.4	86.3
Sesquiterpene hydrocarbons	4.2	20		9.2	1.1	9.5	0.9	3.2	4.4	0.2	1.9	2		2.5	4.5
Oxigenated sesquiterpene	0.4	7.9			0.1	0.4	4.9	0.4	0.3						0.9
Oxygenated diterpene							1.3								
Non-terpene derivatives	0.1		0.9	0.8	0.1		0.8	0.1		0.5	0.6	0.1	0.7	0.1	2
Phenylpropanoids	0.1	12.7	55.6		90.7	90.1									
unknown		0.2		1.7			13.9		0.1						
Total[a]	**98.5**	**99.2**	**99.7**	**97.3**	**100**	**100**	**100**	**98**	**98**	**100**	**99.2**	**100**	**100**	**100**	**97.6**

O.v. – Origanum vulgare; O.b. – Ocimum basilicum; F.v. – Foeniculum vulgare; T.v. – Thymus vulgaris; I.v. – Illicium verum;
S.a. – Syzygium aromaticum; S.s. – Salvia sclarea; O.m. – Origanum majorana; R.o. – Rosmarinus officinalis;
C.a. – Citrus aurantium; C.p. – Citrus paradisi; C.l. – Citrus limon; C.s. – Citrus sinensis; C.b. – Citrus bergamia;
C.c. – Cymbopogon citratus.
[a] All identified compounds were used for calculating the total percentage of each class of constituents.

Table 3. MIC* values of tested essential oils vs. *Penicillium funiculosum* and *Mucor racemosus*, expressed as percentage

	P. funiculosum	*M. racemosus*
Origanum vulgare	1	1.25
Ocimum basilicum	1.25	2.5
Foeniculum vulgare	2.5	5
Thymus vulgaris	2.5	2.5
Illicium verum	5	2.5
Syzygium aromaticum	2.5	2.5
Salvia sclarea	1.25	1.25
Origanum majorana	>5	2.5
Rosmarinus officinalis	5	2.5
Citrus aurantium	>5	5
Citrus paradisi	>5	>5
Citrus limon	>5	>5
Citrus sinensis	2.5	5
Citrus bergamia	2.5	5
Cymbopogon citratus	2.5	1.25
Syzygium aromaticum, Salvia sclarea, Origanum vulgare (1% each)	0.5	0.75
Syzygium aromaticum, Salvia sclarea, Cymbopogon citratus (1% each)	0.5	0.25
Cymbopogon citratus, Thymus vulgaris (1.5% each)	0.75	0.75

*MIC – Minimal Inhibitory Concentration

The most significant finding of this present study was the marked synergistic action observed when EOs were tested as mixtures. M2 in particular appeared to be very interesting, showing a good antifungal activity. This feature allowed the use of a very small amount of EOs (0.5%), with acceptable sensorial properties.

CONCLUSIONS

In conclusion, the results of the present study showed that *S. aromaticum, S. sclarea* and *C. citratus* EOs are characterized by a good antifungal activity, strongly enhanced when combined as a mixture. However, considered that these EOs can be added as natural flavourings in food, further research beyond the present work will be needed to evaluate the organoleptic effects following the application of these natural products in treating cheese rinds.

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