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Control of *Penicillium* sp. on the Surface of Italian Salami Using Essential Oils

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Summary

The goal of this study is to evaluate the *in vitro* effects of rosemary, salvia, oregano and clove oils at volume fractions of 1000, 750, 500, 250, 100, 50, 26, 10 and 5 μL/mL (100, 75, 50, 25, 10, 5, 3, 1 and 0.5 %) on the growth of contaminating fungi in salami. The *in vitro* effect of the oils against fungal growth was indicated by zones of inhibition. Rosemary oil showed an inhibition zone of 9.6 mm only at the maximal volume fraction (1000 μL/mL). Salvia oil showed inhibition zones of 12.2, 11.2 and 10.5 mm only at the three highest fractions tested. Based on the inhibition zones, clove oil at 125 and 250 μL/mL, oregano oil at 250 and 500 μL/mL and a mixture (1:1 by volume) of the two oils at 100 μL/mL were selected to be applied to the surface of salamis. A significant reduction of fungal growth in all of the oil-treated samples was confirmed by visual inspection. A sensory analysis revealed that the samples treated with 125 μL/mL of clove oil or 100 μL/mL of a mixture of oregano and clove oil showed no significant flavour differences compared with the control. Carvacrol and eugenol were the principal compounds in oregano and clove oils, respectively, and were most likely responsible for the antifungal activity.

Key words: essential oil, Italian salami, *Penicillium* sp.

Introduction

Condiments have always been used in the food industry to preserve food. Consumer concerns over excessive use of synthetic additives has motivated the industry to seek for new alternatives to reduce condiment use. In this context, the use of natural products in food preservation is a promising alternative to ensure food quality, extend shelf life and meet the needs and expectations of consumers (*1*). Among industrialised foods, Italian salami has a significant share of the meat market (*2*); however, the characteristic presence of mould is a natural consequence of the technological process of salami manufacturing (*3*).

The growth of filamentous fungi on the surface of salami during ripening is considered a quality factor that should complement the biochemical changes involved in the maturation of the product. Among these biochemical changes are the typical flavour produced by the oxidation of lactate, amino acid degradation and proteolysis, lipolysis, oxygen consumption, protection against light and colonization by undesirable mould (*4*,*5*).

However, many of these fungi can cause changes in colour and flavour and also represent a public health problem because of toxin production (*6*). Contaminating fungi are green, brown or black, colours that are unacceptable to most consumers, and may also have a negative impact on flavour or delay curing time (*7*). Green moulds are usually *Penicillium* and *Aspergillus*, and brown or black moulds are *Cladosporium*, *Alternaria* and *Aspergillus* (*6*).

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Natamycin (pimaricin) is used in the food industry for prevention against harmful fungi and can be sprayed on the surface of cheese and meat products (*3*). However, this treatment adds a significant cost and, at high doses, can cause nausea, vomiting and diarrhoea (*8*).

Essential oils derived from secondary plant metabolism are composed of a mixture of compounds such as monoterpenes, sesquiterpenes, phenyl propanoids, alcohols, aldehydes, esters, ketones, phenols and oxides. These metabolites confer organoleptic characteristics, have therapeutic and pharmacological activity and are also studied for their antimicrobial, antifungal, cytotoxic, anti-inflammatory and insecticide potential (*9*).

Studies on the antifungal activity of essential oils in food are scarce in the literature; however, this issue has aroused enormous interest industrially and commercially. Given the importance of studies related to the manufacture of safe and healthy food products and the emphasis on the use of natural preservatives and additives, this study aims to evaluate the *in vitro* antifungal activity of the oils of rosemary (*Rosmarinus officinalis*), salvia (*Salvia sclarea*), oregano (*Origanum vulgare*) and clove (*Eugenia caryophyllata*). The oils with the highest antifungal activity *in vitro* were tested as curative treatments for controlling harmful fungi on the surface of Italian salamis in an industrial plant. Subsequently, the salamis treated with different volume fractions of the oils were subjected to a sensory analysis of flavour.

Material and Methods

Collection and isolation of a filamentous fungus, an industrial contaminant

A contaminating fungus was collected in the curing room of an Italian salami industrial plant and immediately inoculated on potato dextrose agar (PDA) plates and incubated in a bacteriological oven (model TE-393/2, Tecnal Piracicaba-SP, Brazil) at 30 °C for 72 h. Afterwards, the fungus was transferred to the PDA with chloramphenicol to isolate the contaminant (a green mould, most likely *Penicillium verrucosum*) from the inoculated fungus (a white mould, *P. nalgiovense*), based on its macroscopic characteristics, after incubation at 30 °C for seven days (*10*). The procedure was repeated until the contaminant was isolated.

Characterization and identification of the fungus

Fungal identification was based on classical taxonomy (*6*) *via* morphological examination (macroscopic and microscopic). For microscopic identification, we used a microcultivation technique (*11*). The fungus was inoculated on a slice of agar laid on a sterile glass slide and covered with a sterile coverslip. The slide was then placed in a Petri dish and incubated for 5 days at 25 °C. The coverslip containing adhered hyphae was withdrawn and stained with Cotton Blue dye. The same procedure was adopted to examine spores and hyphae bound to the slide. For a morphological description of *Penicillium*, we used the criteria adopted by Pitt (*12*), Frisvald and Filtenborg (*13*) and Samson and Pitt (*14*). A molecular analysis for their identification was not performed because the scope of the study was to establish the effects of the essential oil on fungi during the industrial production of salami, regardless of contamination.

Chemical composition of the essential oils

The composition of the volatile compounds in the commercial essential oils of rosemary (*Rosmarinus officinalis*), salvia (*Salvia sclarea*), oregano (*Origanum vulgare*) and clove (*Eugenia caryophyllata*), all from Ferquima (Vargem Grande Paulista-SP, Brazil), was characterized by gas chromatography coupled with mass spectrometry (GC--MS; Shimadzu QP5050 A; Kyoto, Japan) using a DB-WAX capillary column (30 m×0.25 mm×0.25 μm; Agilent Technologies, Santa Clara, CA, USA).

The column temperature was programmed to 50 °C for 3 min, increased by 5 °C/min to 130 °C and then by 15 °C/min to 210 °C, and held for 5 min. Helium was used as the carrier gas, and the detector and injector temperatures were 250 °C. A volume of 0.5 mL was injected into the GC-MS system. This device operated at a flow rate of 1 mL/min with an electron impact of 70 eV in split (split ratio 1:3) mode. Compounds were identified by comparing the mass spectra with those in the Wiley library (*15*) using the software provided with the equipment and by comparison with the GC retention times of standard compounds.

Evaluation of the effect of essential oils on the growth of the contaminating fungus

Dilutions of the rosemary (*Rosmarinus officinalis*), salvia (*Salvia sclarea*), oregano (*Origanum vulgare*) and clove (*Eugenia caryophyllata*) oils were prepared in distilled water and 1 % Tween 80, and the oils were tested at volume fractions of 1000, 750, 500, 250, 100, 50, 26, 10 and 5 μL/mL. Each dilution was stirred for 5 min in a shaker tube (Vortex, Fanem, Hamburg, Germany).

The inhibition zone of mycelial growth was evaluated on agar plates according to Silva and Bastos (*16*). A preinoculum (10^6 spores/mL) was added to the PDA medium before solidification at a ratio of 1:10 (10^5 spores/mL) and then poured into sterile Petri dishes. After solidification, four cavities were made with sterile glass tubes (diameter 6 mm).

In each cavity, 50 μL of diluted oil were added, 50 μL of Tween 80 (1 %) were used as a negative control, and 50 μL of the antifungal ketoconazole were used as a standard positive control. The system was then incubated for 72 h at 30 °C. At the end of the incubation period, the diameter of each inhibition zone was measured in millimetres. Six replicates of each experiment were conducted.

Preparation of Italian salami

The salamis were prepared according to the standard formulation of the food industry, which is registered at the Ministry of Agriculture, Livestock and Food Supply of Brazil. The normal procedure in the industrial manufacturing plant was followed until the curing phase. Once the salamis entered the curing room, they were monitored daily. Fifteen days after the placement in the curing

room, the appearance of contaminating fungi on salami that had been spray-coated with test oil was observed.

Application of oils on the tips of salami

The essential oils that showed the best results in the *in vitro* tests were sprayed on the tips of salami previously inoculated with *Penicillium nalgiovense* that showed visual contamination with the green fungus in the industrial plant. The following oils and volume fractions were utilized (10 replicates for each treatment were performed): clove oil at 125 and 250 μL/mL, oregano oil at 250 and 500 μL/mL and a mixture (1:1 by volume) of the two oils at 100 μL/mL. Other 20 ends of salami were left untreated as a negative control for visual and flavour analyses. The dilutions were prepared with distilled water and 1 % Tween 80. The different volume fractions were applied by spraying (100±10) μL/cm^2, without overlapping the spray.

For the visual analysis, dilutions were applied to the tips of the salami (5 cm), where fungal growth generally occurs. For the sensory analysis of flavour, the oil was applied to the entire salami. After the oil was applied, the salamis were stored for 15 days in the curing room and then collected for the visual and flavour analyses.

Sensory characteristics

Forty untrained panellists of both genders, aged between 22 and 50, participated in the sensory analyses. They were all consumers of Italian salami and familiar with it.

Flavour analysis

The flavour analysis was carried out to determine if there was a noticeable difference between the control sample (without oil) and the samples treated with the essential oils as described above. A paired comparison test was employed.

Visual analysis

Visual analysis was performed by comparing the samples to the control (*17*). The presence of fungi in the clip (tip) region of Italian salami treated with 125 and 250 μL/mL of clove oil, 250 and 500 μL/mL of oregano oil or 100 μL/mL of the mixture (1:1 by volume) of the two oils was monitored. The tests were performed individually under white light by comparing the clip region of the control with clip regions of five samples, each coded with a random three-digit number (a sample of each dilution is described in the section Application of oils on the tips of salami).

The panellists were asked to carefully observe the clip region of the coded samples, comparing them with the untreated standard (control), and to evaluate the degree of difference between the standard and the coded samples using a mixed, structured 8-point scale, ranging from no growth (grade 0) to fungal growth equivalent to that on the control sample (grade 8).

Statistical analysis

The *in vitro* evaluation of the antifungal effects of essential oils was performed in six replicates, and the average values were obtained by analysis of variance (ANOVA) and compared with Tukey's test ($p<0.05$) using the SPSS Statistics software package (IBM Corporation, Armonk, NY, USA).

The results of the visual sensory analysis of treated samples were compared with standard sample (control) to test the differences (Dunnett's test with 95 % confidence intervals). The results of the flavour analysis were evaluated with a paired comparison test that analysed the significance of the number of hits (other than the standard) in relation to the number of errors (*17*).

Results and Discussion

Isolation and identification of contaminating fungi

The white mould isolated in the curing room of the industrial plant was identified as *Penicillium* sp., which is most likely *Penicillium nalgiovense* because this fungus had been deliberately inoculated into the product at the plant where the collections were performed.

The green mould was identified as *Penicillium* sp., most likely *Penicillium verrucosum*, but it was not molecularly identified. According to Andersen (*18*) and Bremmelgaard (*19*), these fungi can produce black or brown spots that are not acceptable to consumers, have a negative impact on aroma and flavour and are associated with the production of penicillin, which can cause allergies when consumed in large quantities.

Chemical composition of the essential oils

The essential oil of clove contained the volatile compound eugenol as the major component (89.58 %). This result was similar to the results obtained by Silvestri *et al.* (*20*), who reported that eugenol represented 90.3 % of the volatile compounds. Arenas *et al*. (*21*) analysed clove essential oil and also found eugenol as the principal component but at a lower percentage (60.5 %).

The phenolic monoterpenoid carvacrol, which has known antimicrobial activity, was the major component found in oregano essential oil (60.71 %). Silva *et al*. (*22*) evaluated the essential oil of oregano of five different commercial brands, and all of the chromatograms showed one major peak with a retention time similar to a carvacrol standard; the peaks were identified as carvacrol, which was present in percentages ranging between 61.7 and 93.4 % of the total volatile compounds. Busatta *et al*. (*23*) found only 11.67 % carvacrol in oregano essential oil. The major compounds found in the essential oils of clove and sage were bornyl acetate (39.64 %) and linalool (39.26 %), respectively.

In the 1:1 combination of essential oils, the major component was eugenol (56.42 %), while the second highest component was carvacrol (15.39 %).

Antifungal activity of essential oils

The results of the antifungal activity of the essential oils tested on the isolated green mould (*Penicillium* sp.) are shown in Table 1. Rosemary oil had an inhibitory effect only at 1000 μL/mL, producing an inhibition zone of 9.6 mm.

Table 1. Mean diameter of inhibition zones of the fungus *Penicillium* sp. when using different volume fractions of essential oils

φ/(μL/mL)	*d*/mm			
	Rosemary	Clove	Oregano	Sage
1000	$(9.6\pm0.2)^{aC}$	$(36.7\pm0.2)^{aB}$	$(46.5\pm1.2)^{aA}$	$(1.2\pm0.3)^{aD}$
750	$(0\pm0)^{bD}$	$(36.2\pm0.4)^{aB}$	$(46.2\pm1.0)^{aA}$	$(11.2\pm0.3)^{bC}$
500	$(0\pm0)^{bD}$	$(36.0\pm0.3)^{aB}$	$(39.7\pm0.6)^{bA}$	$(10.5\pm0.2)^{bC}$
250	$(0\pm0)^{bB}$	$(30.7\pm0.3)^{bA}$	$(27.3\pm0.9)^{cA}$	$(0\pm0)^{cB}$
100	$(0\pm0)^{bC}$	$(18.8\pm0.3)^{cB}$	$(24.5\pm0.7)^{cA}$	$(0\pm0)^{cC}$
50	$(0\pm0)^{bC}$	$(16.0\pm0.3)^{dB}$	$(19.3\pm0.7)^{dA}$	$(0\pm0)^{cC}$
26	$(0\pm0)^{bB}$	$(15.8\pm0.3)^{dA}$	$(16.0\pm1.5)^{dA}$	$(0\pm0)^{cB}$
10	$(0\pm0)^{bC}$	$(13.8\pm0.3)^{eA}$	$(5.0\pm0.3)^{eB}$	$(0\pm0)^{cC}$
5	$(0\pm0)^{bB}$	$(10.3\pm0.3)^{fA}$	$(0\pm0)^{fB}$	$(0\pm0)^{cB}$

Mean values followed by the same lower case in the column and the same capital letter in the row do not differ according to the Tukey´s test ($p<0.05$)

Hillen *et al*. (*24*) studied the effect of essential oils extracted from different species, including *Rosmarinus officinalis* (rosemary), on the mycelial growth of fungi (*Alternaria carthami*, *Alternaria* sp. and *Rhizoctonia solani*). Candle and rosemary oils were more effective when aliquots larger than 200 μL were added.

Pereira *et al*. (*25*) also evaluated the *in vitro* efficacy of different essential oils, including rosemary (*Rosmarinus officinalis* L.), on the mycelial growth of the fungi *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus flavus* and *Fusarium* sp. They concluded that the rate of inhibition of the mycelial growth of the fungus *A. ochraceus* by rosemary oil was proportional to the volume fraction tested. Mycelial growth of the fungi *Fusarium* sp. and *A. flavus* was affected at volume fractions of 1500 and 2000 μL/mL, respectively. However, the mycelial growth of the fungus *A. niger* was not affected at any volume fractions tested. Similar results were obtained in this study – rosemary oil showed an inhibition zone only at a volume fraction of 1000 μL/mL.

The results in Table 1 indicate that the sage oil produced an inhibitory effect at volume fractions of 500, 750 and 1000 μL/mL, with inhibition zones of 10.5, 11.2 and 12.2 mm in diameter, respectively. Pozzatti *et al*. (*26*) investigated the antifungal activity of the essential oil of sage and failed to demonstrate any antifungal activity of this oil against various strains of *Candida* spp.

The best results were obtained with oregano oil at higher volume fractions (from 500 to 1000 μL/mL), followed by clove oil. These oils were superior to rosemary and sage oils at all volume fractions, producing zones of inhibition even at the lowest volume fractions tested, thus demonstrating their potential as fungicides (Table 1). A gradual increase in the diameter of the inhibition zones with increasing volume fraction was observed, up to 750 μL/mL of oregano and up to 500 μL/mL of clove oil.

Omidbeygi *et al*. (*27*) tested the antifungal activity of the essential oils of thyme and clove on the development of *Aspergillus*. The essential oils were added at volume fractions of 0, 50, 200, 350 and 500 ppm. The results showed that both essential oils inhibited the growth of the microorganism.

Pereira *et al.* (*25*) evaluated the *in vitro* effects of condiment essential oils, including oregano (*Origanum vulgare* L.), on the mycelial growth of the fungi *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus flavus* and *Fusarium* sp. They reported that all fungi were significantly inhibited by 500 mg/mL of oregano oil with the exception of *A. niger*, which showed a decrease only at a mass concentration of 1000 mg/mL, demonstrating the antifungal power of oregano.

Several studies have shown the capacity of essential oils to inhibit fungal growth; however, the mechanisms by which the oils prevent the growth of microorganisms have rarely been explained. Some authors claim that the antifungal action of the oils is linked to disturbances of the cytoplasmic membrane, changes that cause alterations in membrane proteins, inhibition of the proton motive force, electron flow, active transport and coagulation of cellular contents. Others explain further that the antifungal activity is related to the hydrophobic characteristics of the oil, which helps to break down the cell membrane and mitochondrial lipids, causing changes in the structure and increasing permeability, which in turn causes the leakage of cellular components such as ions (*28–31*).

Analyses of Italian salami

Sensory analysis of flavour

Although essential oils have been extensively studied, their use in foods as antimicrobial substances has been quite limited due to changes in flavour because the doses effective against microorganisms can also change the acceptability of the product. Consequently, a growing demand is present to determine the volume fractions of essential oils that achieve a balance between antimicrobial efficacy and sensory acceptability (*32*). In this regard, a sensory analysis of flavour of Italian salami with the applied different volume fractions of the essential oils of clove and oregano was performed to determine how much oil could be used in the product without producing a significant change of flavour. The results of the flavour analysis are shown in Table 2.

The flavour analysis showed that the samples treated with 125 μL/mL of clove oil or a mixture of clove oil and

Table 2. Paired comparison test for the analysis of flavour of salami treated with different volume fractions of essential oils against the control sample (no essential oil)

Responses of panellists	φ/(μL/mL)				
	Clove		Oregano		Clove/ Oregano
	250	125	500	250	100
Correct answers*	35	19	37	30	21

*For a significant difference ($p<0.05$), minimum of correct answers of the panellists is 26 (*17*).
N(panellists)=40

Table 3. Visual analysis scores of fungal growth on salami treated with different volume fractions of essential oils compared to control sample (without essential oil)

Responses of panellists	φ/(μL/mL)					
	Clove		Oregano		Clove/oregano	Control (without oil)
	250	125	500	250	100	
Average score	(1.07±0.28)^b	(1.60±0.37)^b	(0.77±0.18)^b	(0.73±0.16)^b	(0.73±0.17)^b	(7.63±0.49)^a

Mean values followed by the same letter do not differ significantly from the control sample (standard) with 95 % confidence (Dunnett´s test). Score range: 0=without growth, 2=large reduction of the standard, 4=moderate reduction of the standard, 6=small reduction of the standard, 8=equal to standard.
N(panellists)=40

oregano (1:1) at a dose of 100 μL/mL showed no significant differences in flavour when compared to the control sample ($p>0.05$); that is, the panellists did not notice any flavour differences between the treated samples and the control (without added oil), indicating that the oil could be used at that volume fraction without changing the standard product flavour. A volume fraction of 250 μL/mL changed the flavour of the product, so it was found unacceptable.

Visual analysis of fungal growth on salami

Fig. 1 shows the tip regions of the analysed salami samples. The visual analysis indicated that, compared to the control, there were significant differences among the samples in fungal growth in the region of the salami where the oil was applied. A large reduction in fungal growth was observed in the oil-treated samples with scores lower than 2 (Table 3).

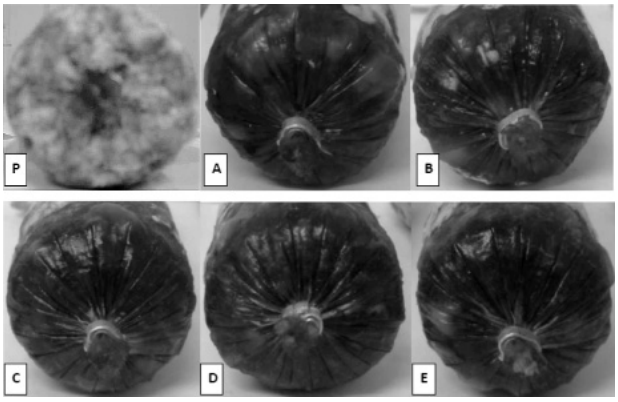

Fig. 1. The tip regions of the six analysed salami samples. P=control sample, and samples treated with: A=clove oil at 250 μL/mL, B=clove oil at 125 μL/mL, C=oregano oil at 500 μL/mL, D=oregano oil at 250 μL/mL, E=mixture of clove and oregano oil (1:1) at 100 μL/mL

It was observed that 125 μL/mL of clove oil or a mixture of 100 μL/mL of clove oil and oregano oil (1:1) had the best overall effect on the appearance and flavour, and can be recommended as a curative treatment to control the growth of undesirable fungi on the surface of salami during the curing process without changing the original flavour characteristics of the product. These results further indicate that the major compound present in the essential oil of oregano, carvacrol, is primarily responsible for the high antifungal activity, although a synergistic effect of eugenol could be present in the treatment that used the mixture of clove oil and oregano oil.

Conclusions

The results of the *in vitro* analyses indicated that rosemary and sage oil did not have a pronounced antifungal effect but that clove and oregano oil were inhibitory even at lower volume fractions. The flavour evaluation showed that 250 μL/mL of clove oil or 500 and 250 μL/mL of oregano oil caused noticeable flavour differences compared to the untreated salami. However, this result does not indicate that the flavour caused by the oil was unacceptable but only that a noticeable difference was present. However, samples treated with 125 μL/mL of clove oil or 100 μL/mL of the mixture of clove and oregano oil (1:1) showed no significant flavour changes when compared to the untreated sample. Visual analysis showed that the sprayed oil inhibited the growth of the fungi and that the inhibition of the fungal growth was visually perceptible when samples with added oil and untreated samples were compared.

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