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Fungal Population Dynamics in Ready-to-eat Salads During a Shelf-life in Italy

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Abstract: The aim of this work was to investigate the fungal population dynamics in ready-to-eat bagged samples of rocket (*Diplotaxis* spp.), lettuce baby leaf (*Lactuca sativa* L.) and "songino" (*Valerianella olitoria* L.) during a shelf-life, in order to evaluate the effects of the storage length and season of production on the spoilage processes. The incidence of toxigenic moulds was particularity studied in order to evaluate a potential production of mycotoxins and allergenic conidia. A total of 900 samples collected from 10 Italian trademarks were analyzed at the 2nd, 5th and 8th day after the packaging in the spring and summer. A very high number of fungi was found and a great variability of moulds and yeasts at the 1st day of sampling was observed. Regarding to season of production, any seasonal effect on the moulds and yeasts has been observed, but the moulds detected belonged to different species in relation to season. Regarding to storage length, the yeasts and moulds did not showed significant variations during a shelf-life. In relation to vegetable species, the lettuce resulted always less contaminated with respect to other salads, and the rocket presented 1-2 Log cfu/g of increasing in the level of moulds. Regarding to fungi species, the yeasts were significantly predominant respect to moulds. Finally, the toxigenic moulds *Aspergillus flavus* and *Penicillium italicum* were found in all the types of salad in the summer, and their growth during the storage at low temperature represented a potential hazard for the mycotoxins and allergenic conidia production in these commodities.

Key words: Fungal population dynamic, ready-to-eat vegetable, shelf-life, toxigenic mould, yeast.

1. Introduction

The ready-to-eat fresh vegetables, also called Minimally Processed Vegetables (MPV) or "IV gamme salads", are raw products that must preserve as much possible the nutritional, sensorial and microbiological qualities of fresh products and that satisfy the consumer request for healthy food with sensorial properties and easy preparation. A very wide range of vegetables are used, both cut and whole. During a production process, the vegetables are selected, washed with sanitizing agents (i.e. sodium hypochlorite, ozone, active oxygen), rinsed, eventually cut, dried and packaged in sealed pouches under modified atmosphere (MAP) or in plastic trays wrapped with an extensible high permeability polymeric film. These vegetables are generally highly perishable during the handling and transportation, and the recommended storage conditions are until to 7-8 days at 6 ± 2 °C. The microbial quality indicators of bagged ready-to-eat salads, fresh vegetables and fruits depends on by several bacteria species (mesophilic aerobic, total and faecal coliforms, *Escherichia coli* O:157, *Salmonella* sp., *Listeria monocytogenes* and *Leuconostoc* sp.) that affect on shelf-life, quality and safety of these commodities. For this reason, these microorganisms have been widely studied and reported in the previous researches [1-9].

Several types of fresh or minimally processed fruit and salad results also more contaminated by fungi (moulds and yeasts) that could be responsible of decay processes, as slime formation, loose firmness and off-odours [10]. An higher level of moulds and yeasts was detected in ready-to-eat vegetables respect to fresh

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uncut fruits, salads and sprouts [11]. Some of these moulds found in fresh vegetables and sprouts contaminate and spoil several plant materials in post-harvest, where they could produce toxic metabolites (mycotoxins) and allergenic conidia during the transport and storage [11]. These moulds, belonging to genera *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium* and *Sclerotinia*, are common plant pathogenic fungi that could be carried by different vectors (i.e. air, irrigation water, insects and infected vegetable sources) from diseased crops to healthy plants, and should contaminate several horticultural crops cultivated under greenhouse (i.e. tomato, lettuce, melon and cucumber) [12].

The lettuce baby leaf or "lattughino" (*Lactuca sativa* L.), "songino" (*Valerianella olitoria* L.) and rocket or "rucola" [(*Diplotaxis erucoides* (L.) DC., *D. muralis* (L.) DC., *D. tenuifolia* (L.) DC.] are commodities particularity appreciated as bagged ready-to-eat salads in the MPV Italian market.

Therefore, the aim of this research work was to investigate the fungal population dynamics (moulds and yeasts) in bagged ready-to-eat samples of *L. sativa*, *V. olitoria* and *Diplotaxis* spp. collected from very important Italian trademarks, in order to evaluate the effects of the storage length and season of production on the spoilage processes. The fungal monitoring and mycological analysis were carried out in two different seasons of production of these commodities during their shelf-life. The incidence of toxigenic moulds was particularity studied, in order to evaluate the potential production of mycotoxins and allergenic conidia during the storage. However, one preliminary study of this topic has already been reported in literature [13].

2. Materials and Methods

2.1 Sampling

Ready-to-eat samples of *L. sativa*, *V. olitoria* and *Diplotaxis* spp. were collected from 10 Italian points of sale. They appeared fresh and without any decay symptom both at moment of colleting and during the monitoring. The samples were immediately transported to laboratory after collecting and maintained at 6 ± 2 °C during the monitoring. In order to establish the effects of storage length, according to label on envelope bag, the fungal analyses were performed at three different times during a shelf-life (2nd, 5th and 8th day after the packaging), by processing of five different lots of samples for each type of salad. Each lot was composed by three samples (60 g each) packaged under MAP in polyethylene bags. In order to evaluate the effects of the seasonal factors, the sampling was carried out in the spring (from March to May) and repeated in the summer (from June to September). A total of 15 samples of each salad type were collected from each trademark and for each season of sampling.

Therefore, a total of 300 samples of lettuce, 300 samples of "songino" and 300 samples of rocket were analyzed by plate count method at the 2nd, 5th and 8th day after the packaging using each bag one time after opening. The 8th day of storage corresponded to last day allowed for the consumption.

2.2 Mycological Analyses and Phytopathogenicity Tests

Thirty grams of each sample were homogenised with 270 mL of 0.1% Bacteriological Peptone-0.85% Sodium Chloride for 2 min using the Stomacher apparatus (mod. 400, Seward, Norfolk, UK). Decimal dilutions of homogenate were performed in distillate sterile water, surface plated in four replications on Yeast extract-Glucose-Chloramphenicol-Agar (YGCA) (Sigma-Aldrich, Milan, Italy) contained in 100 mm Petri plates (100 μL/plate) and incubated at 24 ± 2 °C in the dark under aerobic conditions. The plate count data were recorded at 10th day from the placing and expressed as colony forming units per gram (cfu/g).

The yeast colonies were transferred in 90 mm Petri dishes containing Sabouraud-Dextrose-Agar (SDA) (Biolife, Milan, Italy), incubated at 36 ± 1 °C for 48 hours and identified by BIOLOG YT Micro Plates™ system (AES Laboratory, Milan, Italy) using the

"MicroLog™3" software. The mould colonies were transferred in 90 mm Petri plates containing Potato-Dextrose-Agar (PDA) (Sigma-Aldrich, Milan, Italy), incubated at 25 ± 1 °C for 7-8 days and identified by analysis of morphological and biometrical characters of the colonies, conidiophora and conidia, according to taxonomic keys [14, 15].

The phytopathogenicity tests of three fungal strains (Csp, Asu, Psu), isolated most frequently and belonging to important plant pathogenic fungi, were performed by artificial inoculations on the respective susceptible vegetable hosts. The experimental trials were carried out on orange healthy fruits (*Citrus sinensis* L. Osbeck) cv. Navel and tomato healthy plants (*Lycopersicon esculentum* Mill.) cv. Super Marmande, under controlled conditions of temperature and Relative Humidity (R. H.).

Each orange fruit was washed under running tap water, surface sterilized by dipping in 0.1% Sodium Hypochlorite for 1 min and wounded at four points on surface by sterile scalpel. Each fruit was separately inoculated with a conidia suspension (10^5 conidia/mL) of Asu and Psu (20 μL/wound), obtained from cultures grown on PDA for 10 days at 25 °C, and with 20 μL of sterile distillate water (control sets). The fruit sets were incubated at 26 ± 2 °C and 90 ± 3% R. H. and the symptoms were evaluated on the skin surface at the 7th day from inoculation by "Infection index" (%), Ii = n/N × 100 (n = number of diseased fruits, N = total fruits). The trials, each containing three replications of 10 fruits, were repeated for three times.

Each tomato plant of 45 days-old was sprayed with a conidia suspension (8×10^5 conidia/mL) of Csp obtained from cultures grown on PDA for 20 days at 25 °C, and with sterile distillate water (control sets). The plant sets were maintained at 23 ± 2 °C and 98 ± 2% R. H. and the symptoms were evaluated on the foliar surface after 15 days from inoculation by "Infection index" (%), Ii = n/N × 100 (n = number of diseased plants, N = total plants). The trials were repeated for three times, each one containing three replications of 10 plants.

2.3 Statistical Analysis

Analyses of variance (ANOVA) of data means (both "Plate count" and "Infection index") were carried out with the statistical programme SPSS version 12.0 (Statistics Base™, Chicago, IL, USA). The Tukey's HSD test was applied when ANOVA revealed significant differences ($P < 0.05$) among the means.

3. Results

3.1 Fungal Population Dynamic

The colony forming units per gram data were reported in the Tables 1 and 2, for the moulds and yeasts, at the 2nd, 5th and 8th day after the packaging for each vegetable type. In particular, the tables reports the mould and yeast counts, as mean and as range, evaluated in the spring (Table 1) and in the summer (Table 2).

In general, a very high number of fungi was found and a great variability of mould and yeast count at the 1st day of sampling was observed among all the analyzed samples.

In particular, regarding to storage length, the yeasts and moulds generally did not showed significant variations during the sampling as count mean. Regarding to season of production, any significant difference of mould and yeast count was found among the samples collected in the spring and in the summer at the 2nd day after the packaging. The moulds ranged from 1×10 to 1×10^3 cfu/g in *L. sativa*, from 4×10^2 to 3×10^5 cfu/g in *Diplotaxis* spp., and from to 4×10^2 to 3×10^4 cfu/g in *V. olitoria* in the spring (Table 1). The moulds ranged between 1×10 and 2×10^3 cfu/g in *L. sativa*, 3×10^2 and 6×10^5 cfu/g in *Diplotaxis* spp., and 5×10^2 and 3×10^4 cfu/g in *V. olitoria* in the summer (Table 2). The yeasts ranged from 4×10^2 to 3×10^4 cfu/g in *L. sativa*, from 1×10^3 to 8×10^5 cfu/g in *Diplotaxis* spp. and from 1×10^3 to 2×10^5 cfu/g in *V. olitoria* in the spring (Table 1). In the summer, the yeasts ranged between 5×10^2 and 6×10^4 cfu/g in *L. sativa*, 2×10^3 and 6×10^5 cfu/g in *Diplotaxis* spp.,

Table 1 Monitoring of moulds and yeasts at the 2nd, 5th and 8th day after the packaging on ready-to-eat salads collected in the spring.

Ready-to-eat salads	2nd day				5th day				8th day			
	Moulds		Yeasts		Moulds		Yeasts		Moulds		Yeasts	
	Mean*	Range*	Mean*	Range*	Mean*	Range*	Mean*	Range*	Mean*	Range*	Mean*	Range*
L. sativa **	2×10^2a	1×10 - 1×10^3	4×10^3b	4×10^2 - 3×10^4	4×10^2a	1×10 - 1×10^3	5×10^3 b	5×10^2 - 4×10^4	4×10^2a	1×10 - 1×10^3	8×10^3 b	7×10^2 - 6×10^4
Diplotaxis spp. **	1×10^4 a	4×10^2 - 3×10^5	7×10^4b	1×10^3 - 8×10^5	2×10^4a	5×10^2 - 4×10^5	1×10^5 bc	4×10^3 - 8×10^5	2×10^4a	7×10^2 - 5×10^5	1×10^5 bc	6×10^3 - 8×10^5
V. olitoria **	1×10^3a	4×10^2 - 3×10^4	2×10^4b	1×10^3 - 2×10^5	4×10^3ab	5×10^2 - 4×10^4	8×10^4bc	5×10^3 - 5×10^5	6×10^3ab	8×10^2 - 7×10^4	1×10^5 bc	6×10^3 - 8×10^5

*Colony forming units per gram (cfu/g). **For each row, means followed with the same letter are not statistically different according to Tukey's HSD test ($P < 0.05$). As the same as Table 2.

Table 2 Monitoring of moulds and yeasts at the 2nd, 5th and 8th day after the packaging on ready-to-eat salads sampled in the summer.

Ready-to-eat salads	2nd day				5th day				8th day			
	Moulds		Yeasts		Moulds		Yeasts		Moulds		Yeasts	
	Mean*	Range*	Mean*	Range*	Mean*	Range*	Mean*	Range*	Mean*	Range*	Mean*	Range*
L. sativa **	2×10^2 a	1×10 - 2×10^3	2×10^3b	5×10^2 - 6×10^4	2×10^2 a	1×10 - 2×10^3	2×10^3b	7×10^2 - 8×10^4	2×10^2a	1×10 - 2×10^3	3×10^3b	7×10^2 - 8×10^4
Diplotaxis spp. **	2×10^4 a	3×10^2 - 6×10^5	2×10^4a	2×10^3 - 6×10^5	2×10^4 a	5×10^2 - 4×10^5	1×10^5 b	6×10^3 - 8×10^5	2×10^4a	4×10^2 - 7×10^5	1×10^5b	5×10^3 - 8×10^5
V. olitoria **	2×10^3 a	5×10^2 - 3×10^4	5×10^4b	2×10^3 - 8×10^5	2×10^3a	5×10^2 - 4×10^4	1×10^5 bc	6×10^3 - 8×10^5	2×10^3a	4×10^2 - 7×10^4	1×10^5bc	5×10^3 - 8×10^5

and 2×10^3 and 8×10^5 cfu/g in *V. olitoria* (Table 2). Regarding to fungi genera, the yeasts were significantly predominant with respect to the moulds. In fact, the yeasts mean count ranged from 4×10^3 to 1×10^5 cfu/g in the spring and from 2×10^3 to 1×10^5 cfu/g in the summer. Instead, the filamentous fungi mean count ranged from 2×10^2 to 2×10^4 cfu/g in the spring and from 2×10^2 to 2×10^4 cfu/g in the summer. Finally, in relation to salad type, the lettuce samples resulted always less contaminated by fungi with respect to other vegetables. The rocket samples generally presented 1-2 Log cfu/g of increasing in the level of moulds, as count mean, respect to "songino" and lettuce. Regarding to yeast mean count, generally the data ranged less with respect to mould count.

3.2 Identification of Fungi and Results of Phytopathogenicity Tests

Mixed contaminations by yeasts and moulds belonging to different genera/species were found in the 100% of samples tested. A frequency of the main mould and yeast species isolated from lettuce, "songino" and rocket collected in the spring and summer has been shown in Table 3.

The yeast *Candida oleophila* was obtained from lettuce (74% in the spring and 83% in the summer), rocket (57% in the spring and 62% in the summer) and "songino" (67% in the spring and 77% in the summer). *Rhodotorula* species were found in lettuce (26% in the spring and 17% in the summer), rocket (12% in the spring and 13% in the summer) and "songino" (33% in the spring and 23% in the summer). *Candida edax* was found in the rocket samples with a frequency of 31% and 25% respectively in the spring and summer.

The plant pathogenic fungus *Cladosporium fulvum* (Cke) Cif. (syn. *Passalora fulva*) (Fig. 1) was obtained from lettuce, rocket and "songino" collected in the

Table 3 Species and genera of moulds and yeasts mainly detected during the monitoring of ready-to-eat salad samples collected in the spring and summer.

Ready-to-eat salads	Spring				Summer			
	Moulds		Yeasts		Moulds		Yeasts	
	Species/Genera	Frequency*	Species/Genera	Frequency*	Species/Genera	Frequency*	Species/Genera	Frequency*
L. sativa	*Cladosporium fulvum*	65	*Candida oleophila*	74	*Penicillium italicum*	80	*Candida oleophila*	83
	Sclerotinia sclerotiorum	25	*Rhodotorula* spp.	26	*Aspergillus flavus*	15	*Rhodotorula* spp.	17
	Fusarium solani	10			*Sclerotinia sclerotiorum*	5		
Diplotaxis spp.	*Cladosporium fulvum*	89	*Candida oleophila*	57	*Aspergillus flavus*	48	*Candida oleophila*	62
	Alternaria spp.	7	*Candida edax*	31	*Penicillium italicum*	39	*Candida edax*	25
	Fusarium solani	4	*Rhodotorula* spp.	12	*Alternaria* spp.	8	*Rhodotorula* spp.	13
					Fusarium solani	5		
V. olitoria	*Cladosporium fulvum*	75	*Candida oleophila*	67	*Penicillium italicum*	60	*Candida oleophila*	77
	Alternaria spp.	18	*Rhodotorula* spp.	33	*Aspergillus flavus*	15	*Rhodotorula* spp.	23
	Fusarium solani	7			*Fusarium solani*	25		

*Percentage of colonies grown on YGCA and identified on PDA (for the moulds) and by BIOLOG YT system (for the yeasts).

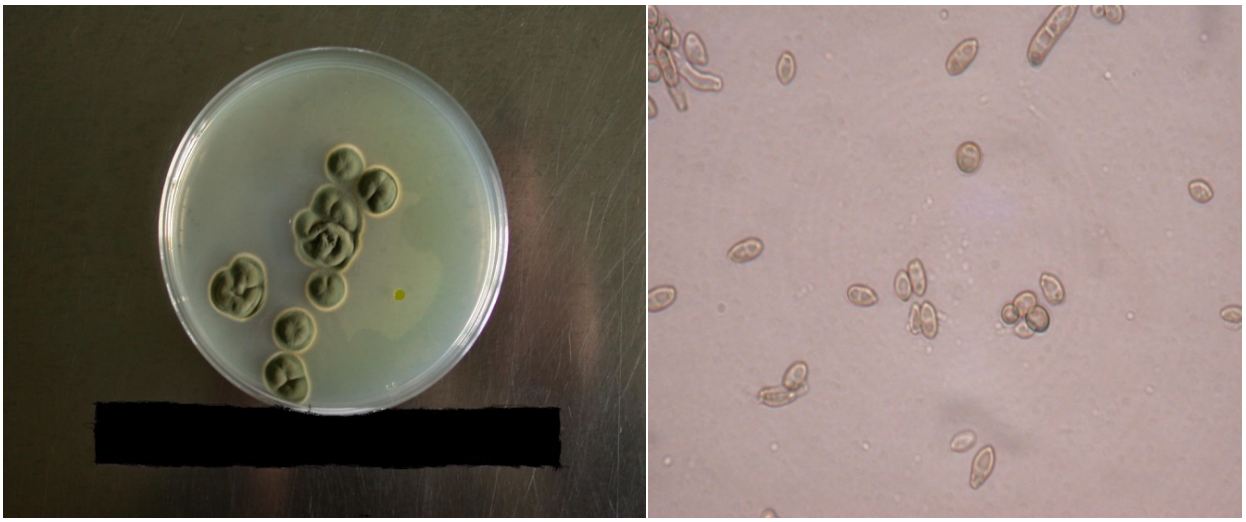

Fig. 1 Colonies (left) and conidia (right) of *Cladosporium fulvum* (syn. *Passalora fulva*) isolated from *Diplotaxis* spp. and grown on PDA.

spring with a frequency of 65%, 89% and 75% respectively. The plant pathogenic fungi belonging to toxigenic genera, as *Aspergillus flavus* (Fig. 2) and *Penicillium italicum* (Fig. 3), were found in lettuce (15% and 80% respectively), "songino" (15% and 60% respectively) and rocket (48% and 39% respectively) sampled in the summer. The remaining fungal colonies were detected in all the salads collected in both seasons. They were belonging to plant pathogenic fungi most important for the Italian horticultural cropping systems, as *Alternaria* spp. (ranging from 7% in rocket to 18% in "songino" collected in the spring), *Fusarium solani* (Mart.) Sacc. (ranging from 4% in rocket collected in the spring to 25% in "songino" sampled in the summer) and *Sclerotinia sclerotiorum* (Lib.) De Bary (found in the lettuce samples with a frequency of 5% in the summer and 25% in the spring).

Finally, the results of phytopathogenicity tests are reported in Table 4. The "Infection index" is resulted of 73%, 53% and 86% respectively for Csp (*C. fulvum*), Asu (*A. flavus*) and Psu (*P. italicum*) strains. All the fungal strains tested *in vivo* for their phytopathogenicity are resulted significantly pathogenic with respect to control set on respective susceptible vegetable host tested.

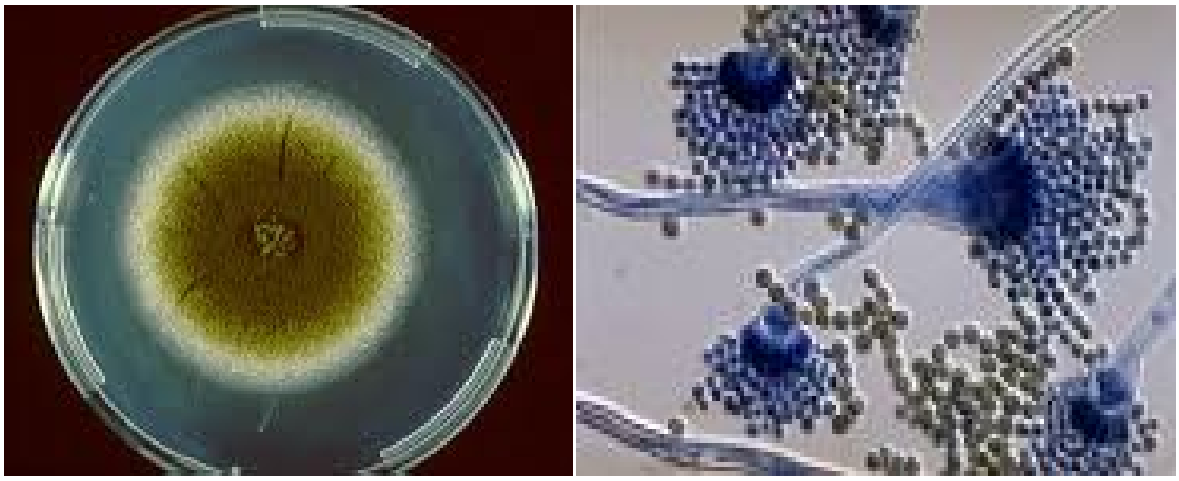

Fig. 2 Colony (left) and conidiophora (right) of *Aspergillus flavus* isolated from *Diplotaxis* spp. and grown on PDA.

Fig. 3 Colonies (left) and conidiophora (right) of *Penicillium italicum* isolated from *Lactuca sativa* baby leaf and grown on PDA.

Table 4 Pathogenicity tests of three fungal strains obtained from ready-to-eat samples of *Lactuca sativa, Valerianella olitoria* and *Diplotaxis* spp.

Fungal strains	Infection index on tomato plants (%)		Infection index on orange fruits (%)	
	Inoculated	Control	Inoculated	Control
Csp	73a	0b		
Asu			53a	0b
Psu			86a	0b

For each row, data followed with different letters are statistically different according to Tukey's HSD test ($P < 0.05$).

4. Discussion and Conclusions

A great variability of mould and yeast count at the 2nd day after the packaging could be related to different origin, retail point and production process of ready-to-eat salads, according to guidelines for the microbiological quality of some ready-to-eat foods sampled at the point of sale throughout the UK [2].

The maintenance of cold chain during the storage has allowed that an initial fungal contamination did not showed significant variations of plate count during a shelf-life, according to the effects of processing and storage conditions on the microbiological quality and shelf-life of ready-to-use salads and minimally processed vegetables [16, 17]. The importance of the maintenance of cold chain during the storage was also

confirmed by studies on the microbial population dynamics in ready-to-eat vegetable salads [18]. Instead, any seasonal effect regarding the mould and yeast counts has been observed in this study, but it is very important to observe that the moulds resulted belonging to several species/genera in relation to different season of MPV production.

The higher yeast count at the 1st day of sampling may be explained by the fact that these commodities, undergone to extensive handling during cutting and packing, could be highly contaminated by the personnel and the utensils used during their processing [11]. Moreover, the significant yeast growth in *Diplotaxis* spp. and *V. olitoria* samples during a shelf-life, could be favoured by increasing of anoxic atmosphere into the bags during the storage, which has been observed for the sliced oranges packed under MAP [19]. The yeast *C. oleophila* is an antagonistic microorganism used in the sustainable agriculture as biological control agent (commercially called as "ASPIRE") against post-harvest spoilage fungi on fruits stored in commercial conditions [20]. It is one common environmental yeast that can be easily isolated from leaves and fruits surface, as lettuce, spinach, strawberries, grapes and kiwifruits. The species of *Rhodotorula* are a pigmented imperfect yeasts easily identifiable by oranges, reds, pinks or yellows colonies grown on SDA. They are common environmental yeasts that sometime may cause red, pink or yellow discolouration in food, and can be isolated from soil, water and air.

Regarding to the filamentous fungi, it is important to observe that *C. fulvum* strain is resulted significantly pathogenic on tomato plants, therefore the ready-to-eat salads contaminated by this fungus could be one potential inocula for the springy tomato crops practised under greenhouse [12]. Instead, the *F. solani* and *S. sclerotiorum* fungi, which may be related to severe spoilage processes in several ready-to-eat vegetables [15, 21], nevertheless have been detected at low frequency on all the samples analyzed.

The other moulds are resulted belonging to several toxigenic genera, as *Alternaria*, *Aspergillus* and *Penicillium*. These toxigenic fungi and their mycotoxins related are most dangerous contaminants of several grain cereals, foods and beverages [22]. In particular, the high number of *A. flavus* and *P. italicum* colonies isolated from all the samples of salad collected in the summer, and their ability to grow at the refrigeration temperature during the storage, represented a potential hazard for the production of mycotoxins and allergenic conidia during a shelf-life, but more detailed studies of this topic are required for determine the concentration of mycotoxins and the amount of allergenic conidia eventually presents in these samples.

Therefore, for improve the quality and safety of these economically important commodities during a shelf-life, it is recommended a careful control of some agricultural practices (i.e., avoiding irrigation with cleaned water and organic fertilizers combined to good sanitization practices against plant pathogenic fungi, toxigenic moulds and human pathogen yeasts), the respect of hygienic conditions during a production process and the maintenance of cold chain during the distribution and storage into a retail points.

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