Preservation of strawberries with an antifungal edible coating using peony extracts in chitosan

Caterina Pagliarulo[1]. Francesca Sansone[2]. Stefania Moccia[3]. Gian Luigi Russo[3]. Rita Patrizia Aquino[2]. Paola Salvatore[4,5]. Michele Di Stasio[3]. Maria Grazia Volpe[3*]

[1]Dipartimento di Scienze e Tecnologie, Università degli Studi del Sannio, via Port'Arsa 11, 82100 Benevento, Italy

[2]Dipartimento di Farmacia, Università di Salerno, Via Ponte Don Melillo, 84084 Fisciano, SA, Italy

[3]Istituto di Scienze dell'Alimentazione-CNR, Via Roma 64, 83100 Avellino, Italy

[4]Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università degli Studi di Napoli "Federico II", via S. Pansini 5, 80131 Napoli, Italy

[5]CEINGE-Biotecnologie Avanzate, Via G. Salvatore 486, 80145 Napoli, Italy

* Corresponding Author:

Dr. Maria Grazia Volpe, Istituto di Scienze dell'Alimentazione- CNR, Via Roma 64 – 83100 Avellino (Italy). Tel: 0039-0825299515; FAX: 0039-0825781585

e-mail: mgvolpe@isa.cnr.it

Abstract

Strawberries represent a valuable source of bioactive compounds including vitamin C, E, β-carotene, and phenolic compounds, but with an extremely short postharvest life. Therefore, the current study was conducted to investigate the effectiveness of an active coating consisting in antifungal microparticles obtained by spray drying of peony extracts (PPR) dispersed in chitosan (Ch) and subsequent addition to polysaccharides gel to slow the fungal attack of small highly perishable fruits, such as strawberries. The results of the antimicrobial assays indicate that the peony extracts in chitosan are capable to effectively counteract the growth of different fungi isolated from deteriorated strawberries. In conclusion, through this treatment with antifungal coating it is possible to prolong the shelf life of delicate fruits such as strawberries, to about 16 d, slowing down the weight loss, affecting the safeguard of important vitamins and antioxidant capacity during storage, without causing any significant alteration of the nutritional and sensorial properties of the product.

Keywords: postharvest; shelf life; *Fragaria*×*ananassa*; fungal growth inhibition; e-nose analyses.

1. Introduction

Strawberries (*Fragaria* × *ananassa*) are some of the most delicate fruits, with an extremely short postharvest life. They are widely consumed, both as fresh fruit and as an ingredient in processed products. Strawberries are a valuable source of bioactive compounds including vitamin C, E, β-carotene, and phenolic compounds (Wojdylo et al. 2009; Cerezo et al. 2010). However, these properties are compromised since they are susceptible to mechanical injury, desiccation, decay and physiological disorders during storage. Moreover, a major problem is gray mold infection, caused by *Botrytis cinerea*, which is one of the most common and serious diseases wherever strawberries are grown (Daugaard 2000; Wszelaki and Mitcham 2003). In effect, this fungus can cause 80-90% losses of flowers and fruit in wet seasons and on unsprayed plants. In addition to the gray mold, numerous other fungal infections can affect the strawberries, like the anthracnose caused by fungi in the genus *Colletotrichum* and the infections caused by some species of *Pichia* (Peres 2005; Wedge 2007).

In order to prevent fungal and insect attack and prolong postharvest shelf life many chemical treatments have been used even if their use should be minimized for food safety. The use of edible coatings which incorporate bioactive molecules may represent a valid and alternative conservation system. The effectiveness of edible coatings for fruits and vegetables depends primarily on the selection of appropriate coating materials that will result in a modified atmosphere within the fruit by controlling respiratory gas exchange (Valencia-Chamorro et al. 2009). What's more, edible coatings can be used as a vehicle to incorporate functional ingredients, such as antioxidants, flavors, colors, antimicrobial agents, and nutraceuticals (Sánchez-González et al. 2010; Diab et al. 2001). Thus, the use of suitable coating techniques can minimize the loss of product by preventing microbial growth and delaying quality loss due to senescence.

The aim of this study was to evaluate the ability of an active coating consisting in antifungal microparticles obtained by spray drying dispersed in biopolymers, to control the fungal growth on strawberries.

2. Materials and methods

2.1. *Materials*

Paeonia rockii (PPR) extract was prepared and characterized as reported by Picerno et al. (2011). Food grade chitosan (Molecular Weight (MW), 100 kDa; degree of deacetylation, 95%), agar (Ag, fine powder, viscosity range 5-50 cps) and all the other chemicals used were of reagent grade and were supplied by Sigma-Aldrich, Milan, Italy.

2.2. *Preparation of Paeonia rockii extracts in chitosan for microbiological assay and growth conditions of fungal microrganisms*

The *Paeonia rockii* extracts in chitosan (Ch-PPR) were dissolved in sterile distilled water in order to obtain a final concentration of 125 μg/μL. To evaluate the antifungal activity of the Ch-PPR, different yeast strains, *Pichia kluyveri*, *Candida valida and Candida pulcherrima,* isolated from strawberries (SFI, strawberries fungal isolates), previously identified, at UOC (Unità Operativa Complessa) of Clinical Microbiology, AOU (Azienda Ospedaliera Universitaria) Federico II of Naples, were used. The identification of the yeast strains isolated from strawberries was performed by mass spectrometry using the Matrix Assisted Laser Desorption/Ionization (MALDI) mass spectometer (Bruker Daltonics, MALDI Biotyper, Fremont, CA, USA), a high-throughput proteomic technique for identification of a variety of bacterial and fungal species (Neville et al. 2011; Sogawa et al. 2011). The microorganisms were cultured in Sabouraud GC (Oxoid, S.p.a., Rodano, Milano, Italy) broth and agar at 25 °C. Fungal strains were maintained at 4 °C on agar media. The isolates were stored frozen at -80 °C in Sabouraud broth supplemented with 10% glycerol (v/v) (Carlo Erba Reagents Srl, Rodano, Milano, Italy) until use and the working cultures were activated in the respective broth at 25 °C for 24-48 h.

2.3. *In vitro antifungal activity assay of the Ch-PPR extracts*

A variation of the agar diffusion method (Bauer et al. 1966), was performed to evaluate the inhibitory activity of the extracts against test microorganisms. Briefly, the fungal strains were grown in Sabouraud broth to an optical density of 0.5 at 600 nm and an aliquot of 200 µL of the fungal suspension was surface-spread on agar media. Paper discs (6 mm in diameter, Oxoid, S.p.a., Rodano, Milano, Italy), impregnated with 8-16 µL of the stock extracts concentrated 125 µg/µL, were positioned on the media using a sterile forceps. Different concentrations of Ch-PPR (1-2 mg/disc) were used to evaluate the antifungal activity. Paper discs impregnated with 8.4 mg/disc of Tioconazole (TCZ) (Pfizer Italia Srl, via Isonzo, Latina, Italy), were used as positive control. Plates were incubated at 25 °C for 2-5 days. The antifungal activity was expressed as the diameter of the inhibition zones produced by the extracts against the test microorganisms. The experiments were repeated three times.

2.4. *Effect of the Ch-PPR extracts on fungal growth and survival*

The susceptibility of the fungal strains to different concentrations of the extracts was determined by the use of the dilution tube method with 1×10^5 CFU/mL as the standard inoculum (Varaldo 2002). The extracts were added in a series of tubes to achieve final concentrations in the range of 0-10 mg/mL. Subsequently, the tubes were incubated at 25 °C for 48-72 h. The isolates were also tested with TCZ as positive control and with the extraction buffer (MeOH) as negative control. After incubation, optical density at 600 nm was measured from each tube and an aliquot was surfaces-spread onto Sabouraud agar plates in duplicate. Plates were then incubated for 48-120 h to evaluate the viable count. The minimum fungicidal concentration (MFC) was defined as the minimum extract concentration that killed 99% of fungi from the initial inoculums. Minimum inhibitory concentration (MIC) was assigned to lowest concentration of extract which prevented fungal growth.

2.5. *Preparation of coating solution*

The encapsulation of a polar extract of PPR by spray drying technique using chitosan (C) as polymeric matrix was performed as reported by Sansone et al. (2014). Food-grade agar was used for the coating formulations. Preliminary tests using 0.5, 1 and 2% (p/v) agar were carried out to determine the optimal coating formulation. The best results in terms of gel strength was obtained with 1% agar and applied to the following coating formulations. According to Erkan et al. (2011), 0.25% of PPR in chitosan (Ch-PPR) was used in the coating formulation. Gel solution was prepared by mixing 1 g of agar with 100 mL of distilled water and stirred at a temperature of 100 °C until the mixture became clear. Before gelation (about 30 °C), 0.25% of Ch-PPR was mixed with the prepared solution and stirred thoroughly. Strawberries were divided into three groups: control (C), uncoated strawberries; coated strawberries (Ag), fruits dipped for few seconds in 1% agar solution; active coated strawberries (Ch-PPR/Ag), fruits dipped for few seconds in 1% agar solution containing 0.25% Ch-PPR. All samples were then stored at 4 °C.

2.6. *Treatment of strawberries*

Strawberries cultivar *Candonga* were harvested at the red ripe stage at "Amendola Carmine" farm (Battipaglia, Salerno, Italy) and transported to the laboratory within 2 h in a refrigerated box. The fruits were sorted to eliminate damaged, shriveled, and unripe berries and selected for uniform size and color. 25 strawberries, weighing about 300 g, were dipped in the coating solutions and put in polypropylene (PP) containers covered with a microperforated sheet, and stored at 4 °C for sixteen days. The same procedure, except for the dipping was followed for the control samples. The analyses were performed on days 0, 2, 4, 8, 12 and 16 , using three replicates /treatment.

2.7. *Colony count of moulds and yeasts*

The procedure of the Total Viable Count (TVC) of fungal colonies on agar plates was used for the quantitative estimation of the level of fungal microorganisms present in strawberry samples, according to the microbiological methods provided by ISO 21527-1:2008 and according to the European Regulations 852/2004. Samples (25 g) were homogenized in 250 mL of 0.1% peptone solution (Oxoid, S.p.a., Rodano, Milano, Italy) using a paddle peristaltic homogenizer Stomacher 400 Circulator (Seward LTD, UK) and an aliquot of the appropriate serial dilutions was transferred to DRBC agar (Oxoid, S.p.a., Rodano, Milano, Italy), in duplicate. Plates were then incubated at 25 °C for 5 days and viable count was performed. Results were expressed as colony forming units per gram of fruit (CFU/g) (Mali and Grossmann 2003; Yurdugül 2005). Fungal colony count was performed at the beginning of the experiment (day 0) and 4, 8 and 16 days after treatment.

2.8. *Weight Loss*

Weight loss was registered on three replicates/treatment every 2 d during storage, using an electtronic weighing scale (Gibertini elettronica, type Europe 500; Milan, Italy).

2.9. *Total Ascorbic acid concentration*

The content of ascorbic acid in strawberries was determined by Iodometric titration (Moor et al. 2005). Iodine solution (0.05 M) was prepared in the following way: Weigh 20 g of potassium iodide into a 100 mL beaker. Weigh 13 g of iodine (Applichem) and add it into the same beaker. Add a few mL of distilled water and swirl for a few minutes until iodine is dissolved. Transfer iodine solution to a 1 L volumetric flask, making sure to rinse all traces of solution into the volumetric flask using distilled water. Make the solution up to the 1 L mark with distilled water. 25 g of berries were mixed with 100 mL of 6 % solution of oxalic acid and homogenized for 1 minute. The sample was filtered and 2 mL

of 1 % solution of starch paste solution 1% in water (Carlo Erba Reagents, Rodano (Mi), Italy) were added to10 mL of filtrate. This mixture was titrated until change of color, which did not disappear during 30 seconds. The content of ascorbic acid (mg per 100 g of berries) was calculated from the following equation:

$$C = 400 \times \frac{V_{sample}}{V_{standard}}$$

Where

C: content of ascorbic acid;

V_{sample}: volume of the iodine solution titrated in a sample, mL;

$V_{standard:}$ volume of the iodine solution titrated in a standard solution, mL;

2.10. *Extraction of total phenolics*

Extraction of phenolics was carried out by using the method of Terefe et al. (2009). Briefly, the berries were homogenized in the extraction solution (70% methanol/1% HCl) using a blender (Philips, Type HR1652/90, 600 W). Subsequently, 50 g of the homogenate were mixed with 100 mL of the extraction solution and the mixture was sonicated for 16 minutes with an ultrasonic processor (Lind Sonic ISCO) at a temperature below 30 °C. The extraction was repeated twice with extracts kept for 16 hours at 4 °C on a platform shaker with orbital motion (VDRL type 711). After centrifugation for 15 minutes at 6000 rpm and filtration using Whatman number 4 filter paper, total polyphenol content of the filtrate was determined. All analysis were performed in triplicate. The concentration of total phenolics in the extracts was measured using the Folin-Ciocalteu method and gallic acid (Sigma-Aldrich, Italy) as a standard (Singleton and Rossi 1965). 1.58 mL of water and 100 μL of Folin-Ciocalteu reagent (Sigma-Aldrich) were added to 20 μL of sample. After 3 min, 300 μL of 75 g/L of Na_2CO_3 were added and the samples were incubated for 30 min at room temperature. The absorbance was read at 765 nm with an UV/VIS Spectrophotometer (Beckman Coulter, mod. DU 730, Brea, CA,

USA) against a blank. The solutions without added extract used as blank samples. The total phenolics content was calculated from a standard curve prepared using gallic acid and expressed as mg gallic acid equivalent (GAE)/100 g fresh weight.

2.11. *Antioxidant Capacity by the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) Radical Inhibition Assay*

The antioxidant capacity was determined by the DPPH radical scavenging method (da Silva Pinto et al. 2008). A 250 μL aliquot of the aqueous strawberry extract (5 g of homogenate strawberries in 100 mL distilled water) was mixed with 1.25 mL of DPPH (60 μM in ethanol). The mixture was stirred and placed in the dark for 30 min. Then the absorbance was measured at 517 nm (Beckman Coulter, mod. DU 730, Brea, CA, USA). The solutions without added extract used as control. The readings were compared to controls and the percentage of inhibition was calculated by the equation

$$\%\ \boldsymbol{inhibition} = \frac{\boldsymbol{Abs_{control} - Abs_{extract}}}{\boldsymbol{Abs_{control}}} \times \mathbf{100}$$

2.12. *Electronic nose*

A commercial portable electronic nose (PEN 3), including the Win Muster software for data mining, Airsense Analytics Inc. (Schwerin, Germany), was used to analyze the olfactory quality of strawberry samples. The instrument was equipped with an array of 10 metal oxide semiconductors (MOS) type chemical sensor. The sensor response was expressed as resistivity (Ω). The high temperature (200–500 °C) allowed no interference from water and fast response and recovery times (Kohl 1992). The detection limit of hot sensors was 1 mg kg^{-1} (Torri et al. 2008). Five grams of each sample were taken from the inside of the fruits were placed in an air tight 20 mL glass vial, sealed with a PTFE/silicone septum and a screw cap, stored at 25 °C for 1 h to equilibrate and analysed at the same temperature. The results have been displayed in a correlation matrix by using the Win Muster software.

2.13. *Statistical analysis*

The results were expressed as mean ± standard deviation (SD) of tree independent measure for each sample, with n=3, calculated using MS-Excel. One Way Analysis of Variance (ANOVA) was used to estimate significant differences in Total Ascorbic acid concentration, Total phenolics Content and DPPH radical inhibition assay. To isolate the group or groups that differ from the others multiple comparison procedure (Fisher LSD Method) was used. For the antioxidant activity, after an analysis of variance, to isolate the group or groups that differ from the control was used a multiple comparison procedure. (Dunn's Method). Statistical analysis was performed using STATISTICA (data analysis software system), version 10 (StatSoft, Inc., Tulsa, OK, USA).

For the electronic nose analysis ten independent measures were performed for each sample.

Correlation Matrix is used to investigate the dependence between multiple variables at the same time showing the severability of classes. Values are in the range of 0 and 1, low values show a rather bad severability and the classes are difficult to discriminate by the measurement, while high values, near 1, indicate a good severability of classes. Values of discrimination index $P \geq 0.95$ were considered significant as previously reported (Laurienzo et al. 2013).

3. Results and discussion

3.1. *In vitro antifungal activity assay of the Ch-PPR extracts*

The antifungal activity of the Ch-PPR coating was tested against two different fungal isolates obtained from deteriorated strawberries. The diameters of the inhibition zones (clear zones around discs) of fungal growth exerted by the extracts towards the challenged microorganisms are shown in Table 1. TCZ, used as a positive control, showed antifungal efficacy against all the isolates tested, with inhibition zones of 26±1.41 and 29±1.41 mm against *Candida valida* and *Pichia kluyveri*, respectively. The Ch-PPR extracts, at concentrations of 1 and 2 mg/disc led to the formation of a clear inhibition zone of growth of 16.75±1.06 and 19±1.41 mm, respectively against *Candida valida*. Similarly, the inhibition zone for *Pichia kluyveri* growth was of 24.5±0.70 mm and 27.25±0.35 mm, at concentrations of 1 and 2 mg/disc, respectively (Table 1).

The values of the diameters of inhibition zones of fungal growth obtained with the Ch-PPR extracts are high, comprised between 19±1.41 and 27.25±0.35 mm at concentrations of 2 mg/disc, and these are close to values obtained with the tioconazole (26±1.41 and 29±1.41 mm), effective antifungal at broad-spectrum, used as a control. Therefore, the growth inhibition of yeasts isolated from impaired strawberries is appreciable and concentration-dependent.

This result indicates that the Ch-PPR effectively counteracted *in vitro*, the growth of fungi isolated from deteriorated strawberries. Our results are in agreement with other studies based on the antimicrobial activity of chitosan and peony extracts (Roller and Covill 1999; Tapia et al. 2009; Palmeira-de-Oliveira et al. 2010; Ngan et al. 2012).

3.2. *Effects of extracts on fungal growth and survival*

Quantitative evaluation of the antifungal activity of Ch-PPR was carried out against *Pichia kluyveri*, *Candida valida* and *Candida pulcherrima*, all SFI, by the use of the dilution tube method, according to the CLSI (Clinical and Laboratory Standards Institute) guidelines (CLSI, 2012).

The results of the sensitivity assays are shown in Table 2. In the present study all the tested isolates were sensitive to TCZ. The fungistatic and fungicidal effects of Ch-PPR were greater against *Pichia kluyveri*. The values of MIC, MBC and MFC of different natural extracts determined in different studies significantly vary (Roller and Covill 1999; McCarrell et al. 2008; Chatterjee et al. 2014; Sansone et al. 2014; Pagliarulo et al. 2016). This variability is not surprising, considered it is typically recorded even with conventional antimicrobials against all isolates (EUCAST data 2013).

In addition, the effect on growth and survival of *P. kluyveri*, the most sensitive strain to Ch-PPR, was evaluated using the chitosan and the peony extracts separately. In the presence of chitosan, the fungal growth was inhibited at concentration of 8 µg/µL, while the fungicidal effect was obtained at concentration of 30 µg/µL. When the peony extracts are tested alone, concentrations higher than 20 and 60 µg/µL are needed to observe growth inhibition and to obtain fungicidal effect against *P. kluyveri*, respectively. These results, compared with those shown in Table 2, clearly indicate that the antifungal activity of peony extracts and of chitosan is less when they are used separately against the yeast isolate. Instead, the antifungal activity is greater when they are used together in the form of Ch-PPR extracts (Table 2). The precise synergistic effect is to be investigated.

The traditional use of *Paeonia rockii* is thought to be related to its phenolic compounds. Indeed, a recent systematic phytochemical and biological study (Picerno et al. 2011) on *Paeonia rockii* showed that 5-butylhydroxy-γ–lactone, ethyl-arabinopyranose, polyphenolic compounds, mono- and tri-terpenes are the main constituents of a crude polar methanol-soluble extract. The whole dried extract and its portion soluble in n-BuOH have the ability to scavenge free-radicals and to inhibit *Candida albicans* growth (Picerno et al. 2011). According to Choubey et al. (2015), these activities are mainly

due to the presence of gallic acid derivatives which are individually able to scavenge free-radicals and to inhibit fungal growth. In addition, the simultaneous presence of various compounds, such as paeoniflorin in combination with gallic acid, could enhance the activity of the extracts suggesting a potential synergy in the action or enhancement of bioavailability of the antimicrobial molecules (Picerno et al. 2011).

The antimicrobial properties of the chitosan are dependent on many factors, including the environmental conditions such as pH, type of microorganism, and neighboring components; and its structural conditions such as molecular weight, degree of deacetylation, derivative form, its concentration, and original source (Hosseinnejada and Jafari 2016). The exact mechanism of the antimicrobial action of chitin, chitosan and their derivatives is not clearly understood but different mechanisms have been proposed (Kong et al. 2010). The chitosan interacts with the plasma membrane of the microrganisms to alter cell permeability (Benhamou et al. 1996; Jung et al. 1999; Xing et al. 2009). The chitosan as a chelating agent binding with trace metals inhibits the microbial growth and bacterial toxins (Cuero et al. 1991). It also activates several defense processes in the host tissues (El-Ghaouth et al. 1992), acts as a water-binding agent and inhibits various enzymes. The interaction of diffused hydrolysis products of the chitosan with microbial DNA leads to the inhibition of the mRNA and protein synthesis (Hadwiger et al. 1985; Devlieghere et al 2004).

Considering the fungistatic and fungicidal activities of the Ch-PPR on the strawberries fungal isolates, preservation tests were carried out using an active coating on strawberry samples by evaluation of microbiological, chemical and physical-chemical parameters in a time period of 16 days.

3.3. *Fungal colony counts on agar*

In order to evaluate and to compare the microbiological shelf life of the strawberry samples untreated versus the strawberry samples treated with active coating a basis of Ch-PPR, the procedure of the

TVC of fungal colonies on agar plates was carried out. Figure 1 shows the results of the analyses carried out. During the 16 days of storage, the total fungal count on treated strawberries was significantly reduced compared to untreated samples. Therefore, the edible antimicrobial active coating was effective in reducing the native fungal microflora on strawberries enhancing the microbiological shelf-life of the fruits.

3.4. *Weight loss*

During storage, the fruits tend to lose a substantial amount of water with decreases of weight decidedly high. This leads to a qualitative decay as early as the first week of storage.

Weight loss of fruits during storage is not only a factor affecting product quality, but it also represents a problem of economic impact for producing companies. The preservation of fruit in storage systems suitable to extend the shelf life, is a task of central importance in the distribution chain. It can be observed in Figure 2, the best results were obtained for Ch-PPR/Ag and Ag; in fact the weight of the strawberries in the two typologies of coating decreased slowly during 16 days of storage and after 16 days the strawberries in Edible Active Coating lost only 3,4 % and in coating the 8,9%. Strawberries used as control, on the contrary, lost 23% of weight at the end of experiment (16 days), highlighting that the coating is a valid system for keeping under control this parameter. Also the results of Colla et al. (2006) confirm that some optimized formulation coating significantly reduced the weight loss of strawberries during the storage.

3.5. *Total Ascorbic acid concentration*

Strawberries are an important source of ascorbic acid. The recommended daily intake (RDA) for ascorbic acid (AA) is 60 mg/day for adults; this dose can be satisfied with an average of 100 g of strawberries per day (Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids

2000); therefore, it is important to safeguard AA concentration during storage. We evaluated the AA concentration during shelf life for all investigated systems (Figure 3). The differences in the mean values among the groups indicate that there is a statistically significant difference $P \leq 0.001$; infact Fisher LSD Method show that both Ch-PPR/Ag (LSD = 6.267, $P<0.001$) and Ag (LSD = 6.267, $P<0.005$) are statistically different respect to control samples.

The concentration of AA in the *Candonga* cultivar at t = 0 was 66.76 mg/100 g of fresh weight (fw). The experimental data are in agreement with the literature, where AA concentration ranged between 43.0 and 90.5 mg/100 g fw in different cultivars (Cordenunsi et al. 2003; Corral-Aguayo et al. 2008). During storage, the total AA content decreased regularly in the control strawberries (C) to 54.58 mg/100 g fw at 16 days, while it increased in Ch-PPR/Ag and Ag samples to 68.58 and 66.41 mg/100 g fw, respectively.

In literature, changes in AA concentrations temperature-dependent are reported: low temperatures (about 1 °C) prevent the oxidation of total AA, compared to higher temperatures (10- 20 °C) (Shin et al. 2007; Nunes et al. 1998). Moreover, according to Lee and Kader (2000), the loss of AA was related to fruit dehydration. In our experiments carried out at a constant temperature the data obtained showed that a greater loss of AA was associated with strawberries used as control (25 % loss), due to fruit senescence while the coating and the active coating maintain fruits still in the ripening stage (Esteves et al. 1984).

3.6. *Total phenolics Content (TPC)*

The phenolic composition of vegetable is extremely heterogeneous, depending by several factors that determine the biosynthesis in plants. These factors are: geographical origin (latitude and altitude), climatic conditions (photoperiod and degree of UV radiation, temperature, water availability, etc.), environmental (pollutants are inducers of oxidative stress), genotype, cultivation methods, stage of

development at the time of collection, storage conditions, biotic factors (attacks by insects or other pathogens) (Van der Sluis et al. 2001; Bolling et al. 2010).

Fig. 4 shows that TPC was affected by time of storage and preservation methods. The multiple comparison procedures (Fisher LSD Method) show that both Ch-PPR/Ag (LSD = 12.647, $P \leq 0.003$) and Ag (LSD = 12.647, $P \leq 0.011$) are statistically different, compared to control samples. TPC values ranged from a minimum of 123.76 mg GAE/100g fw at t0 to a maximum of 154.17 mg GAE/100g fw for Ch-PPR/Ag after 16 days of storage. Relatively to the phenolics concentration of fruits just harvested, our results are in agreement with the literature, which reports values of TPC in the range of 96-330 mg/100 g fw, depending on cultivars analyzed and extraction method employed (Wang and Lin 2000; Proteggente et al. 2002; Meyers et al. 2003; Klopotek et al. 2005; Terefe et al. 2009). As an example, Meyers et al. (2003) reported values of TPC of approximately 273 mg GAE/100 g wet weight for cultivars *Earliglow*, *Evangeline* and *Annapolis*, while an average of 202 mg GAE/100 g was observed for *Mesabi*, *Jewel* and *Allstar*.

During storage, the polyphenols concentration of the strawberries can increase if a suitable preservation system is employed. The stability of polyphenols in fruits is influenced by many external factors such as light and oxygen. Compared to raw fruit, there was an increase of polyphenols concentration in Ch-PPR/Ag sample during 16 days of storage because the active coating is oxygen-proof (Siah et al. 2011).

3.7. *Antioxidant Capacity by the DPPH Radical Inhibition Assay*

The antioxidant activity of the phenolic extracts was evaluated by the DPPH radical inhibition assay (Figure 5). In this study, we found that storage in active coating significantly affected the antioxidant activity of strawberry fruits ($P<0.05$). Infact the antioxidant activity of the phenolic extract at time zero was equal to 79.90%; instead at the end of storage the antioxidant activity of Ch-PPR/Ag sample

was 77.86%, those of Ag sample was 74.07% and for C sample the value was 64.55%, significantly lower than Ch-PPR/Ag.

Literature data report that the antioxidant activity of strawberries depends on cultivars, phenolic compounds present (da Silva Pinto et al. 2008) and a combination of different molecules with synergic and antagonistic effects (Hassimotto et al. 2005). The decrease of the antioxidant activity in control sample is related to the decrease of total ascorbic acid and phenolic compounds content.

3.8. *Electronic nose analysis*

The correlation matrices of the scores obtained by the Electronic Nose among the samples groups are shown in Table 3a, 3b and 3c. It is possible to observe increasing values of discrimination indexes of days 4, 8, 12 and 16 respect to days 0 in the three correlation matrices. The values of UC classes are higher respect to the values of coated class samples. In particular Ch-PPR/Ag samples at 4 days show a value of 0.490, lower then 0.5 (Table 3c) indicating that the olfactory characteristics do not undergo substantial changes (see statistical analysis section) and the significant level has been reached after 16 days. Control and Ag samples reach significance level more quickly, at 12 days (Table 3a and 3b). It is possible to hypothesized that treated samples retain olfactory characteristics of fresh product better than controls during storage and Ch-PPR/Ag coating is the best formulation to retain olfactory characteristics.

4. Conclusions

In our study, the data obtained from different microbial assays indicate that peony extracts in chitosan possess a clear antifungal activity, as evidenced by the net inhibitory effect on fungal growth and survival of different yeasts isolated from deteriorated strawberries. In fact, the microbiological tests showed a high antifungal activity of the edible active coating at relatively low concentration of peony

extract. Antimicrobial assays showed that the combination of natural extracts and chitosan were able to inhibit the growth and kill microorganisms contaminants of the strawberries with efficacy comparable to that of toxic antifungal agents conventionally used in therapy. The application of edible coating additives with the antimicrobial particles let to preserve perishable fruits and their olfactory properties for several days without using the chemicals preservatives.

To date, different edible active coatings have been employed to improve the quality and shelf life of strawberries, but the edible active coating, a basis of Ch-PPR, tested by us, considering its remarkable effectiveness and security, is very promising for using in the preservation of strawberries.

Acknowledgments

The investigation was supported by grants "Cluster AGRIFOOD-SOSTENIBILITA' DELLA FILIERA AGROALIMENTARE (SO.FI.A)".

Figures legend

Fig. 1 Fungal growth in strawberries samples untreated (●) and treated (▲) with Ch-PPR

Fig. 2 Effect of different preservation systems on the weight loss of control, active coated and coated strawberries during 16 days of storage. The values represent the mean of six measurements.

Fig. 3 Effect of preservation conditions on total Ascorbic Acid concentration of fresh strawberris stored at +4 °C up to 16 days: C, CH-PPR/Ag and Ag. Data is the average of six measurements, and the vertical bars indicate standard deviation. For each storage period, columns with different letters are different by Fisher's protected LSD test ($P < 0.05$) applied after an ANOVA

Fig. 4 Total phenolics (gallic acid equivalent on a fresh weight basis, mg/ 100g) of strawberries samples, C, Ch-PPR/Ag and Ag stored for up to 16 days. For each storage period, columns with different letters are different by Fisher's protected LSD test ($P < 0.05$) applied after an ANOVA

Fig. 5 Antioxidant activity of the phenolic extracts, evaluated by the DPPH radical inhibition assay. Data is the average of six measurements, and the vertical bars indicate standard deviation. To isolate the group or groups that differ from the others a multiple comparison procedure was used (Dunn's Method) applied after an ANOVA; for each storage period, columns with different letters are different ($P < 0.05$)

References

Bauer, A. W., Kirby, W. M., Sherris, J. C., & Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology,* 45(4), 493–496.

Benhamou, N. (1996). Elicitor-induced plant defense pathways. *Trends in Plant Science*, 1(7), 233–240.

Bolling, B., Dolnikowski, G., Blumberg, J., & Chen, C.Y. (2010). Polyphenol content and antioxidant activity of California almonds depend on cultivar and harvest year. *Food Chemistry,* 122, 819–25.

Cerezo, A. B., Cuevas, E. P., Winterhalter, P., García-Parrilla, M. C., & Troncoso, M. A. (2010). Isolation, identification, and antioxidant activity of anthocyanin compounds in Camarosa strawberry. *Food Chemistry*, 123, 574–582.

Chatterjee, S., Chatterjee, B. P. , & Guha, A. K. (2014). A study on antifungal activity of water-soluble chitosan against *Macrophomina phaseolina*. *International Journal of Biological Macromolecules,* 67, 452-7.

Choubey, S., Varughese, L. R., Kumar, V., & Beniwal, V. (2015). Medicinal importance of gallic acid and its ester derivatives: a patent review. *Pharmaceutical Patent Analyst*, 4(4), 305-15.

CLSI (Clinical and Laboratory Standards Institute). (2012). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved Standard-Ninth Edition, M07-A9, Vol. 32 No. 2. URL http://clsi.org/.

Colla, E., Sobral, P. J. A. & Menegalli, F. C. (2006). Effect of composite edible coating from *Amaranthus cruentus* flour and stearic acid on refrigerated strawberry (*Fragaria ananassa*) quality. *Latin American Applied Research*, 36, 249-254.

Cordenunsi, B. R., Nascimento, J. R. O., & Lajolo, F. M. (2003). Physico-chemical changes related to quality of five strawberry fruit cultivars during cool-storage. *Food Chemistry*, 83, 167–173.

Corral-Aguayo, R. D., Yahia, E. M., Carrillo-Lopez, A., & Gonzalez.Aguilar, G. (2008). Correlation between Some Nutritional Components and the Total Antioxidant Capacity Measured with Six Different Assays in Eight Horticultural Crops. *Journal of Agricultural and Food Chemistry*, 56, 10498–10504.

Cuero, R. G., Osuji, G., & Washington, A. (1991). N-carboxymethylchitosan inhibition of aflatoxin production: Role of zinc. *Biotechnology Letters*, 13(6), 441–444.

da Silva Pinto, M., Kwon, Y. I., Apostolidis, E., Lajolo, F. M., Genovese, M. I., & Shetty, K. (2008). Functionality of Bioactive Compounds in Brazilian Strawberry (*Fragaria* × *ananassa* Duch.) Cultivars: Evaluation of Hyperglycemia and Hypertension. *Journal of Agricultural and Food Chemistry,* 56(12), 4386–4392.

Daugaard, H. (2000). Effect of cultural methods on the occurrence of grey mould (*Botrytis cinerea* Pers.) in strawberries. *Biological Agriculture and Horticulture*, 18, 77–83.

Devlieghere, F., Vermeulen, A., & Debevere, J. (2004). Chitosan: antimicrobial activity, interactions with food components and applicability as a coating on fruit and vegetables. *Food Microbiology*, 21, 703–714.

Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids. (2000). Washington, DC: The National Academies Press.

Diab, T., Biliaderis, C. G., Gerasopoulos, D., & Sfakiotakis, E. (2001). Physicochemical properties and application of pullulan edible films and coatings in fruits preservation. *Journal of the Science of Food and Agriculture*, 81, 988–1000.

El-Ghaouth, E. A., Arul, J., Asselin, A., & Benhamou, N. (1992). Antifungal activity of chitosan on post-harvest pathogens: Induction of morphological and cytological alterations in *Rhizopus stolonifer*. *Mycological Research*, 96, 769–779.

Erkan, N., Tosun S. Y., Ulusoy, S., & Üretener, G. (2011). The use of thyme and laurel essential oil treatments to extend the shelf life of bluefish (*Pomatomus saltatrix*) during storage in ice. *Journal of Consumer Protection and Food Safety*, 6, 39–48.

Esteves, M. T., Carvalho, V. D., De Chitarra, M. I. F., Chitarra, A. B. & Paula, M. B. (1984). Characteristics of fruits of six guava (*Psidium guajava* L.) cultivars during ripening. II.Vitamin C and tannins contents. In *Anais do VII Congresso Brasileiro de Fruticultura.* pp.. 490–500, Empresa Catarinense de Pesquisa Agropecuaria, Florianopolis, Brazil.

EUCAST data 2013. URL http://www.eucast.org/

Hadwiger, L. A., Kendra, D. F., Fristensky, B. W., & Wagoner, W. (1985*)*. Chitosan Both Activates Genes in Plants and Inhibits RNA Synthesis in Fungi. *In: Chitin in Nature and Technology*, Plenumx Press, New York, NY, pp. 209–222.

Hassimotto, N. M. A., Genovese, M. I., & Lajolo, F. M. (2005). Antioxidant activity of dietary fruits, vegetables and commercial frozen fruit pulps. *Journal of Agricultural and Food Chemistry*, 53, 2928-2935.

Hosseinnejad, M., & Jafari, S. M. (2016). Evaluation of different factors affecting antimicrobial properties of chitosan. *International Journal of Biological Macromolecules*, 85, 467–475.

ISO 21527-1:2008. Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of yeasts and moulds - Part 1: Colony count technique in products with water activity greater than 0,95.

http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=32875.

Jung, B., Kim, C., Choi, K., Lee, Y. M., & Kim, J. (1999). Preparation of amphiphilic chitosan and their antimicrobial activities. *Journal of Applied Polymer Science*, 72, 1713–1719.

Klopotek, Y., Otto, K., & Bohm, V. (2005). Processing Strawberries to Different Products Alters Contents of Vitamin C, Total Phenolics, Total Anthocyanins, and Antioxidant Capacity. *Journal of Agricultural and Food Chemistry*, 53, 5640-5646.

Kohl, D. (1992). Fundamentals and recent developments of homogeneous semiconducting sensors. In: Gardner, J.W., Bartlett, P.N. (Eds.), *Sensors and Sensory Systems for an Electronic Nose*. Kluwer Academic Publishers, Dordrecht, pp. 53–76.

Kong, M., Chen, X. G., Xing, K., & Park, H. J. (2010). Antimicrobial properties of chitosan and mode of action: a state of the art review. *International Journal of Food Microbiology*, 144, 51–63.

Laurienzo, P., Cammarota, G., Di Stasio, M., Gentile G., Laurino C., & Volpe, M. G. (2013). Microstructure and olfactory quality of apples de-hydrated by innovative technologies. *Journal of Food Engineering*, 116, 689-694.

Lee, S. K., & Kader, A. A. (2000). Preharvest and postharvest factors influencing vitamin C content of horticultural crops. *Postharvest Biology and Technology*, 20, 207-220.

Mali, S., & Grossmann, M. V. E. (2003). Effects of yam starch films on storability and quality of fresh strawberries (*Fragaria ananassa*). *Journal of Agricultural and Food Chemistry*, 51, 7005–11.

McCarrell, E., Gould, S., Fielder, M., Kelly, A., El Sankary, W., & Naughton, D. (2008). Antimicrobial activities of pomegranate rind extracts: enhancement by addition of metal salts and vitamin C. *BMC Complementary and Alternative Medicine*, 8, 64.

Meyers, K. J., Watkins, C. B., Pritts, M. P., & Liu, R. H. (2003). Antioxidant and antiproliferative activities of strawberries. *Journal of Agricultural and Food Chemistry*, 51, 6887–6892.

Moor, U., Karp, K., Põldma, P., & Pae, A. (2005). Cultural systems affect content of anthocyanins and vitamin C in strawberry fruits. *European Journal of Horticultural Science*, 70(4), 195-201.

Neville, S. A., Lecordier, A., Ziochos, H., Chater, M. J., Gosbell, I. B., Maley, M. W., & van Hal, S. J. (2011). Utility of matrix-assisted laser desorption ionization-time of flight mass spectrometry following introduction for routine laboratory bacterial identification. *Journal of Clinical Microbiology*, 49(8), 2980-2984.

Ngan, L. T, Moon, J. K., Shibamoto, T., & Ahn, Y. J. (2012). Growth-inhibiting, bactericidal, and urease inhibitory effects of *Paeonia lactiflora* root constituents and related compounds on antibiotic-susceptible and -resistant strains of *Helicobacter pylori*. *Journal of Agricultural and Food Chemistry*, 60(36), 9062-73.

Nunes, M. C. N., Brecht, J. K., Morais, A. M. M. B., & Sargent, S. A. (1998). Controlling Temperature and Water Loss to Maintain Ascorbic Acid Levels in Strawberries During Postharvest Handling. *Journal of Food Science*, 63(6), 1033-1036.

Pagliarulo, C., De Vito, V., Picariello, G., Colicchio, R., Moccia, S., Pastore, G., Salvatore, P., Volpe, M. G. (2016). Inhibitory effect of pomegranate (*Punica granatum L.*) polyphenol extracts on the bacterial growth and survival of clinical isolates of pathogenic *Staphylococcus aureus* and *Escherichia coli*. *Food Chemistry*, 190, 824–831.

Palmeira-de-Oliveira, A., Ribeiro, M. P., Palmeira-de-Oliveira, R., Gaspar, C., Costa-de-Oliveira, S., Correia, I. J., et al. (2010). Anti-Candida activity of a chitosan hydrogel: mechanism of action and cytotoxicity profile. *Gynecolgic and Obstetric Investigation*, 70(4), 322-327.

Peres, N. A., Timmer, L. W., Adaskaveg, J. E. & Correl, J. C. (2005). Lifestyles of *Colletotrichum acutatum*. *Plant Disease*, 8, 784-796.

Picerno, P., Mencherini, T., Sansone, F., Del Gaudio, P., Granata, I., Porta, A., & Aquino, R. P. (2011). Screening of a polar extract of *Paeonia rockii*: Composition and antioxidant and antifungal activities. *Journal of Ethnopharmacology*, 138, 705-712.

Proteggente, A. R., Pannala, A. S., Paganga, G., van Buren, L., Wagner, E., Wiseman, S., et al. (2002). The antioxidant activity of regularly consumed fruit and vegetables reflects their phenolic and vitamin C composition. *Free Radical Research*, 36, 217-233.

Regulation (EC) 852/2004 on the hygiene of foodstuffs.

Roller, S., & Covill, N. (1999). The antifungal properties of chitosan in laboratory media and apple juice. *International Journal of Food Microbiology*, 47, 67-77.

Sánchez-González, L., González-Martínez, C., Chiralt, A., & Cháfer, M.. (2010). Physical and antimicrobial properties of chitosan–tea tree essential oil composite films. *Journal of Food Engineering*, 98, 443–452.

Sansone, F., Picerno, P., Mencherini, T., Porta, A., Lauro, M. R., Russo, P., & Aquino, R. P. (2014). Technological properties and enhancement of antifungal activity of a *Paeonia rockii* extract encapsulated in a chitosan-based matrix**.** *Journal of Food Engineering*, 120, 260-267.

Shin, Y., Liu, R. H., Nock, J. F., Holliday, D., & Christopher, B. W. (2007). Temperature and relative humidity effects on quality, total ascorbic acid, phenolics and flavonoid concentrations, and antioxidant activity of strawberry. *Postharvest Biology and Technology*, 45, 349-357.

Siah, W. M., Faridah, H., Rahimah, M. Z., Mohd Tahir, S. & Mohd Zain, D. (2011). Effects of packaging materials and storage on total phenolic content and antioxidant activity of *Centella asiatica* drinks. *Journal of Tropical Agriculture and Food Science*, 39(1), 1-7.

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *American Journal of Enology and Viticulture*, 16, 144-158.

Sogawa, K., Watanabe, M., Sato, K., Segawa, S., Ishii, C., Miyabe, A., Murata, S., Saito, T., & Nomura, F. (2011). Use of the MALDI BioTyper system with MALDI-TOF mass spectrometry for rapid identification of microorganisms. *Analytical and Bioanalytical Chemistry*, 400(7), 1905-1911.

Tapia, C. P., Soto, D. M., Vergara, L. G, Alburquerque, C. O., Maccioni, A. R, Matamata, A. M, et al. (2009). Antifungal effect of high molecular weight chitosan on *Candida* spp isolated from clinical samples. *Revista Chilena de Infectologia*, 26(6), 515-519.

Terefe, N. S., Kerstin, M., Lloyd, S., & Cornelis, V. (2009). Combined high pressure-mild temperature processing for optimal retention of physical and nutritional quality of strawberries (*Fragaria×ananassa*). *Innovative Food Science and Emerging Technologies*, 10(3), 297–307.

Torri, L., Piergiovanni, L., & Caldiroli, E. (2008). Odour investigation of granular polyolefins for flexible food packaging using a sensory panel and an electronic nose. *Food Additive and Contaminants*, 25(4), 490-502.

Valencia-Chamorro, S. A., Pérez-Gago, M. B., del Río, M. A., Palou, L. L. (2009). Effect of antifungal hydroxypropyl methylcellulose (HPMC)–lipid edible composite coatings on postharvest decay development and quality attributes of cold-stored 'Valencia' oranges. *Postharvest Biology and Technology*, 54 (2), 72–79.

Van der Sluis, A. A., Dekker, M., De Jager, A., Jongen, W. M. F. (2001). Activity and concentration of polyphenolic antioxidants in apple: effect of cultivar, harvest year, and storage conditions. *Journal of Agricultural and Food Chemistry*, 49, 3606–13.

Varaldo, P. E. (2002). Antimicrobial resistance and susceptibility testing: an evergreen topic. *The Journal of Antimicrobial Chemotherapy*, 50(1), 1-4.

Wang, S. Y., & Lin, H. S. (2000). Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental stage. *Journal of Agricultural and Food Chemistry*, 48(2), 140-146.

Wedge, D. E., Smith, B. J., Quebedeaux, .J. P. & Constantin, R. J. (2007). Fungicide management strategies for control of strawberry fruit rot diseases in Louisiana and Mississippi. *Crop Protection Journal*, 26, 1449–1458.

Wojdylo, A., Figiel, A., & Oszmianski, J. (2009). Effect of Drying Methods with the Application of Vacuum Microwaves on the Bioactive Compounds, Color, and Antioxidant Activity of Strawberry Fruits. *Journal of Agricultural and Food Chemistry,* 57(4), 1337–1343.

Wszelaki, A. L.; & Mitcham, E. J. (2003). Effect of combinations of hot water dips, biological control and controlled atmospheres for control of gray mold on harvested strawberries. *Postharvest Biology and Technol*ogy, 27, 255–264.

Xing, K., Chen, X. G., Liu, C. S., Cha, D. S., Park, H. J. (2009). Oleoyl-chitosan nanoparticles inhibits *Escherichia coli* and *Staphylococcus aureus* by damaging the cell membrane and putative binding to extracellular or intracellular targets. *International Journal Food Microbiology,* 132, 127–133.

Yurdugül, S. (2005). Preservation of quinces by the combination of an edible coating material, semperfresh, ascorbic acid and cold storage. *European Food Research and Technology*, 220,579-586.