Effect of chestnut tannin extract (*Castanea sativa* Miller) on the proliferation of *Cladosporium cladosporioides* on sheep cheese rind during the ripening

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Abstract

Two water solutions of chestnut tannin extract i) 200 gL^{-1} (CHE200), ii) 400 gL^{-1} (CHE400), and the powder of CHE (CHEP) were tested in a cheese making trial in which 60 cheese units were allotted to 5 experimental groups (each of 12 cheeses; control 1, C1: without any treatment; control 2, C2: treated with a silver ion solution; treated cheeses LCHE200, LCHE400, LCHEP: treated respectively with CHE200, CHE400, CHEP). The cheeses were ripened in a room polluted with *C. cladosporioides* with the aim to create the condition for the proliferation of this fungus on the cheeses. The results indicated that CHE at the concentration of 200 gL^{-1} is capable of inhibiting completely *C. cladosporioides* proliferation, avoiding spoilage of the sheep cheese.

1. Introduction

Cheese ripening is a complex process of biochemical changes involving several agents such as endogenous milk enzymes, starter or non-starter microbiota, and secondary microorganisms as moulds which can contaminate the cheese surface from the environment or can be purposely inoculated. For instance, in mould ripened cheeses, several starters such as *Penicillium camamberti* and *Penicillium roqueforti*, are inoculated specifically with the aim to improve the development of flavor and aroma, by degrading proteins and lipids or fermenting sugars. Other moulds such as *Rhizomucor miehei*, *Rhizomucor pusilus*, *Cryphonectria parasitica* can be used to improve milk coagulation or accelerate the ripening of cheese (Cousin, 2003). In contrast, moulds such as *Mucor fuscus* (forming black cottony spots), *Thamnidium elegans* (forming gray spots) and *Cladosporium cladosporioides* (forming black tight spots) or other potentially toxigenic species within the *Penicillium*, *Aspergillus* and *Fusarium* genera are very undesirable because their activity results in a deterioration of cheese esthetical, nutritional and organoleptic quality (Galli Volonterio, 2005; Kure, Wasteson, Brendehaug & Skaar, 2001; Montagna et al., 2004). Colonization by strains belonging to the genus *Cladosporium* is responsible for the damage to the surface of sheep and cow cheese, making it spotted and impossible the sell (Kure, Skaar & Brendehaug, 2004; Gripon, 1993; Cousin, 2003; Delavenne et al., 2011). It is not possible to remove this mould from the rind by wetting or dry-brushing the cheese because it forms black tight and insightful spots, formed by the fungal mycelium. Moreover, *Cladosporium* is one of the most common airborne moulds found in indoor and outdoor environments of dairy processing plants. Its spores are important aeroallergenes and the prolonged exposure to elevate spore concentrations can induce a chronic allergy and asthma (Zeng, Westermark, Rasmuson-Lestander & Wang, 2006). Also, the presence of this microorganism in cheese is not desirable from consumer health perspective, being a potential risk of mycotoxin formation (Havaux, Zeine, Dits & Denis, 2005).

In order to avoid the colonization by strains belonging to the genus *Cladosporium* during the ripening, the surface of cheeses is normally covered with polymeric solutions enriched with antifungal agents such as Pimaricin or antimicrobial solutions containing silver ions. However, these approaches are discouraged because microorganisms can develop a resistance and these substances are pollutants to the environment and problematic for their disposal.

Tannins are polyphenols and secondary metabolites, synthesized by plants against pathogens. These compounds are very heterogeneous and characterized by antimicrobial properties (Landete, 2011; Okuda, 2005). Chestnut wood from the *Castanea sativa* Miller plant species found in the entire Mediterranean area, contains hydrolysable tannins. Industries producing building materials use large quantities of wood that is chipped. If the wood is obtained from chestnut tree, the chip (before its processing) can be steam distilled to obtain a hydrolysable tannin extract characterized by a high level of pureness and high solubility (Romani, Campo, & Pinelli, 2013). In the literature the antimicrobial properties of chestnut tannin extract (CHE) are known, CHE is commonly used in the wine industry as a clarifying agent, color stabilizer and to bring body and flavor to wines lacking in taste and texture (Tosi et al. 2013; Obreque-Slíer, Peña-Neira, López-Solís, Ramírez-Escudero & Zamora-Marín, 2009). Hence, the aim of this trial was to test the efficiency of CHE against *C. cladosporioides* proliferation on sheep cheese rind during ripening, as an alternative treatment to the use of antifungal agents or chemical solutions.

2. Materials and methods

2.1 Isolation and identification of *C. cladosporioides* from cheeses.

During the ripening (sheep cheese, produced by Caseificio il Forteto, Vicchio del Mugello, Firenze, Italy), 10 cheeses with black spots (Figure 1A and 1B), formed by the fungal mycelium presumably belonging to *C. cladosporioides*, were placed in a separate sterile bag and immediately transferred to laboratory. In a sterile chamber, one of the spots from each cheese unit, was isolated by streaking of the mould on to Petri dishes containing Malt Extract Agar (MEA; cod CM0059B; Oxoid s.p.a., Rodano, Italy). The dishes were incubated at 25°C for 7 days (Vaughan, Buzzini, & Clementi, 2008; Wistreich, 1997). Basing on morphology and colour of the colony and microscopic observation, thirty strains (three from each cheese unit) presumably identifiable as *C. cladosporioides*, were isolated as pure cultures.

For identification of the thirty strains, fungal colonies derived from single spores were cultivated on Potato Dextrose Agar (PDA; cod P2182 , Sigma-Aldrich, St. Louis, MO, USA). After 7 days of growth at 25°C in the dark, mycelium (about 0.2 g of fresh weight) was harvested by scraping the surface with a scalpel. Genomic DNA was extracted from the mycelium using the DNeasy Plant Mini Kit (cod 69104, Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The quality of genomic DNA was determined by electrophoresis on agarose gel 1% (cod 16500-500, Ultra Pure Agarose, Invitrogen, Carlsbad, CA) and the quantification using a spectrophotometer ND1000 Nano Drop (Thermo Fisher Scientific, Wilmington, DE, USA). The ITS (Internal Transcribed Spacer) region was amplified using the primers ITS1 (5'- TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCTTAT TGA TAT GC-3') as described by White, Bruns, Lee & Taylor (1990). Ten PCR products were purified with the QIA quick PCR purification Kit (cod 28104 Qiagen) and stored at -20°C until used for sequencing. The sequence of the amplicons was performed by Eurofins Genomics Ebersberg, Germany. The sequence obtained in FASTA format were compared with those deposited in GenBank DNA database (http://www.ncbi.nlm.nih.gov/) using the basicBLAST search tools (Altschul, Gish, Miller, Myers & Lipman, 1990).

2.2 *In vitro* test on effect of CHE on the *C. cladosporioides* growth.

Commercial CHE powder (CHEP) from chestnut wood extraction (Saviotan Feed ©; provided by Gruppo Mauro Saviola, Mantova, Italy) containing 750 g of equivalent tannic acid/kg on dry matter (DM) basis was used for antimicrobial protection of the cheese rind. CHE extract was composed of only hydrolysable tannins and the gas-chromatographic profile is available in Campo et al. (2012). The tannic equivalents of the CHE extract have been determined according to Burns (1963). Six water solutions of CHE were prepared 35 gL^{-1} (CHE35), 50 gL^{-1} (CHE50), 75 gL^{-1} (CHE75), 100 gL^{-1} (CHE100), 200 gL^{-1} (CHE200) and 400 gL^{-1} (CHE400) of CHEP in sterile distilled water, mixing slowly to avoid the foaming. All equipment was sterilized before the preparation of the solutions. The *in vitro* antimicrobial ability of the different CHE solutions and of CHEP against *C. cladosporioides* has been tested by means of Kirby-Bauer antibiogram test (Bauer, Kirby, Sherris & Turck, 1966). An aliquot of the mould previously developed on the Petri dish (see 2.1 section) was distributed on 9 cm diameter Petri dish surfaces (n=40) containing MEA medium. Immediately, in the centre of each plates, a 10 mm diameter paper blotting diskette (Sigma-Aldrich), saturated with a CHE solutions or CHEP or with sterile water as control (C) was positioned (8 plates per treatment). All plates were incubated at 25 °C for 7 days. Then, the different diameter halos of mould growth inhibition around the diskettes or the absence of inhibition zones were determined.

2.3 Cheese-making trial.

The cheese making was done at an industrial dairy processing plant located in Tuscany (Caseificio il Forteto, Vicchio del Mugello, Florence, Italy). The cheeses used for the experiment were produced using the same bulk milk, originating from 3 flocks.

86 The procedure of cheese making was: after the pasteurization process, the sheep milk was maintained to 35 °C and rennet (liquid animal rennet, cod
87 25556, Danisco, Copenhagen, Denmark) was added to gel the milk (120 mL of rennet each quintal of milk corresponding to a rennet/ milk ratio of
88 20ml of rennet each quintal of milk; rennet strength, 1:15000). Then, the milk was inoculated a with starter culture (lyophilized culture) which was
89 composed of strains of *Lactococcus lactis* subsp. *lactis* and of *Lactococcus lactis* subsp. *cremoris* (Lyofast CMS DOP 020, Sacco s.r.l., Italy; 0.05 g
90 of lyophilized starter per each litre of milk). The final counts of total starter bacteria was 10^6 CFU per litre of milk. After the milk clotting (30 min),
91 the curd was cut to the size of a hazelnut and the vat temperature was kept at 35 °C. Curds were placed into 2 kg molds. Sixty-three cheeses were
92 produced, and placed into a thermostatic chamber at 30°C for 6h and turned five times. Then, held for 24 h at 20 °C, after which cheeses were placed
93 in a salt solution (NaCl, 19% w/v) at 15°C for 24 h and finally transferred to a ripening room and analyzed for mould and yeast (in triplicates). The
94 choice of the treatments was performed on the base of the *in vitro* trial results (see 2.2 section). After 5h from salting, 60 cheeses were randomly
95 allotted in 5 groups and treated as follow: 12 units were not treated with any solution (Control 1, hereinafter will be called C1), 12 units were treated
96 with a commercial polymeric solution containing silver ions (250 mg L^{-1} AgPOLYMER C_2, AgPolymer s.a.s, Torino, IT), as antimicrobial (Control
97 2, hereinafter will be called C2), 12 units were treated with the CHE200 solution (LCHE200), 12 units were treated with the CHE400 solution
98 (hereinafter will be called LCHE400) and 12 units were treated directly with pure powder CHEP (hereinafter will be called LCHEP). All solutions
99 were applied by dipping the cheeses in the aqueous suspensions (60 sec) while the powder was directly applied on the surface (until complete coverage
100 of the surface; about 0.5 mm of thickness).

101 Twenty four 14cm diameter Petri dishes containing *C. cladosporioides*, (strain Cc1; mycelium and spores; see 2.1 section), grown on MEA medium
102 were placed (one Petri disk/m^3) in the ripening room (3mx2mx4m) in order to contaminate the ventilated room with mould spores. The Petri dishes

remained during the entire ripening period. A homogeneous spread of spores was achieved throughout the room by a ventilation system with an internal recycle of air.

After the rind treatment, the cheeses were ripened for 100 days at 11-12 °C and sampled at 15, 40, 80, 100 days from cheese making (3 cheeses at each time period per thesis) to monitor the fungal colonization. At 100^{th} day, cheeses were analysed for proximate analysis, physical observation and fatty acid profile.

2.4 Microbiological characterization of cheeses.

At each sampling time, 3 cheeses were placed in to sterile bags and immediately transferred to the laboratory for the microbiological analysis. A one hundred square centimetres sterile silhouette was placed on the upper surface of each cheese (in the centre) and the designated area was scrubbed thoroughly with a cotton swab moistened with saline solution (NaCl, 9 gL^{-1} w/v). The cotton swab was then reinserted into the tube containing 5mL of the same solution (NaCl, 9 gL^{-1} w/v), stirred and stored in the refrigerator until the determination of mould and yeast concentrations. Serial 10-fold dilutions were then performed on the saline solutions and 100 µl of each suspension were plated (5 replicates), using the spread plate method on MEA (Vaughan et al., 2008; Wistreich, 1997). Moulds and yeasts, recognizable by morphology and colour of colony and microscopic observation, were counted after incubation at 25 °C under aerobic conditions for 7 days and 3 days respectively. In order to normalize the data distribution, the results were expressed as Log_{10} of mean colony-forming unit per cm^2 (cfu cm^{-2}).

A representative number of mould and yeast colonies from MEA medium was randomly picked from different plates, purified and characterized by molecular methods in order to identify at species level the dominant microbial population. After a microscopic observation, 10 mould colonies and 10 yeast colonies from each treatment and from each sampling time, were picked from the Petri plates; the isolates were 200 moulds and 200 yeasts.

Also, from samples C1 after 80 and 100 days of cheese ripening, 40 mould colonies having black colour were picked from the Petri plates.

2.5 Identification of mould and yeast isolates.

Two hundred mould isolates, having white colonies, were identified and 20 were purified and sequenced as stated in section 2.1. Similarly, 40 mould isolates, having black colour, were identified and 10 of them were purified and sequenced as stated in section 2.1. The selecting criteria for the purification and sequencing of isolates were in accordance with Venturi, Guerrini & Vincenzini (2012).

Two hundred yeast isolates were identified by PCR-RFLP analysis of the rDNA-ITS (Internal Transcribed Spacer) region as according to Granchi, Bosco, Messini & Vincenzini (1999), using *Hae*III, *Hinf*I and *Cfo*I (Fermentas Inc, Burlington, Ontario, Canada) as restriction endonucleases. The restriction fragments were separated (at 100 volt for 2.5 h) on a 20 g L^{-1} (w/v) agarose gel (Lonza Group Ltd, Basel, Switzerland), containing ethidium bromide (Sigma-Aldrich) and TEB buffer (1 M Tris, 10 mM EDTA, 0.9 M boric acid, pH 8.3; Sigma-Aldrich). The profiles were observed by UV transillumination and compared with those reported in the literature (Esteve-Zarzoso, Belloch, Uruburu, & Querol, 1999; Granchi et al., 1999; Pulvirenti, Solieri, Gullo De Vero & Giudici, 2004). To confirm the identification obtained by PCR-RFLP analysis of the rDNA-ITS region, PCR products of 20 representative strains were purified using Nucleo Spin Extract II (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and sent to BMR Genomics (Padua, Italy) for sequencing. The selecting criteria for the purification and sequencing of isolates were in accordance with Venturi

et al., (2012). The sequences obtained in FASTA format were compared with those deposited in GenBank DNA database (http://www.ncbi.nlm.nih.gov/; Altschul et al., 1990).

2.6 Chemical and physical characterization of the cheeses.

Dry matter (DM) and crude protein (CP) were determined according to the 930.15 and 976.06 AOAC procedures (1995) while cheese fat content was analysed according to Gerber-Van Gulik (ISO, 1975). The method of Thiobarbituric Acid (TBARS) was adopted to monitor the oxidative status of cheese samples according to Patton & Kruiz (1951). Fatty acids from cheese samples were extracted and analysed for their profile according to published methods (Buccioni et al. 2010; 2012). All results for fatty acid composition are expressed as mg g^{-1} of lipids.

Because tannins have the characteristic of colouring and of permeating the organic matter, each cheese unit has been evaluated also for the colour of the crust and of the texture.

2.7 Statistical analysis

The microbial data were checked for normal distribution by Shapiro-Wilk test (SAS, 1999) and normalized by $\log_{10}$ transformation. Hence, normalized data of microbial abundance were processed using a linear model with two fixed factor (treatment and time of ripening) and their interaction (SAS, 1999).

$$y_{ij} = \mu + T_i + R_j + (Ti \times R_j) + e_{ij} \qquad (1)$$

where y_{ij} is the observation; μ is the overall mean; T_i the treatment (i = 1 to 5), R_j the time of ripening (j=1 o 5), Ti x R_j their interaction and e_{ij} the residual error. Multiple comparisons among means were performed using the Tukey's test.

Data of cheese chemical composition and fatty acid profile were analysed using the one way ANOVA (SAS, 1999).

3 Results

3.1 Isolation and identification of *C. cladosporioides* from cheeses.

All thirty isolates from the cheeses were identified by amplification of the ITS region and rITS amplicon size was 550 bp (Figure 2); ten PCR products of the thirty were sequenced and their sequences matched those of *C. cladosporioides* in GenBank (EU030342) with 100% identity scores. Hence, all isolates were identified as *C. cladosporioides* (Fresen) G. A. de Vries. The molecular approach used for strain characterization did not permit the identification of isolates at strain level. Therefore, since all the isolates showed a similarity with 100% identity scores, the selection was random and the strain Cc1 was chosen for in vitro tests.

3.2 *In vitro* test on effect of CHE solutions and powder (CHEP) on the *C. cladosporioides* growth.

161 The ability of the CHE solutions and powder to avoid the development of *C. cladosporioides* Cc1 growth was evaluated using Kirby Bauer test (Figure
162 3). CHE35 produced no inhibition on the *C. cladosporioides* mould growth. In contrast, all other solutions and the powder induced inhibition. The
163 diameter of the inhibition halo increased with the concentration of CHE solutions: with CHE50 it was 15.0 mm, while with CHE75 and CHE100 it
164 was 20.0 mm. In particular, the greatest inhibition halo was produced by CHE200 and CHE400 and the CHEP, that was 30.0 mm. Cc1 was used to
165 contaminate the ripening room.

166

167 3.3 Identification of moulds and yeasts in cheese rind.

168 During ripening moulds and yeasts proliferated on the cheese rind. Hence, the identification of these microorganisms was carried out. Two hundred
169 mould isolates, presumably identifiable as *Penicillium* spp. base on microscopic observation and colony colour and morphology, were identified by
170 amplification of the ITS region. The rITS amplicons were 580 bp (Figure 2). Twenty PCR products, of the two hundred, were sequenced and their
171 sequences matched those of *P. commune* in GenBank (DQ132814) with 99% identity scores. Hence, all isolates were identified as *Penicillium*
172 *commune* (Thom).

173 Forty mould isolates, presumably identifiable as *C. cladosporioides* based on microscopic observation and colony colour and morphology, were
174 identified by amplification of the ITS region. The rITS amplicons were 550 bp (Figure 2). Ten PCR product, of the forty, were sequenced and their
175 sequences matched those of *C. cladosporioides* in GenBank (EU030342) with 100% identity scores. Hence, all isolates were identified as
176 *Cladosporium cladosporioides* (Fresen) G. A. de Vries.

All isolates of yeasts (total number 200), identified by amplification of the ITS region, were characterized by rITS amplicons having a size of 656 bp (Figure 2). In order to obtained species specific PCR-RFLP patterns (Granchi et al., 1999) amplicons of all the isolates were digested with 3 restriction enzymes. The rITS restriction profiles were in base pairs: 400, 120, 75 for *Hae*III; 315, 315 for *Hinf*I; 295, 280 for *Cfo*I, suggesting the attribution of the isolates to the species *Debaryomyces hansenii*. ITS sequences of 20 strains, from 200, matched those of *D. hansenii* in GenBank (FR686595) with 100% identity scores. Hence, all yeast isolates, were identified as *D. hansenii.*

3.4 Mould and yeast development on the cheeses surfaces during ripening of cheese.

The total number of moulds on cheeses before treatments was 1.9 cfu cm^{-2} (0.3 $\log_{10}$).

At 15^{th} day on LCHE400, moulds were lowest compared to those found on LCHE200, LCHEP, C1 and C2. Moreover, at this point of sampling the moulds detected on C2 were higher than those found in C1 and similar to those found on LCHE200.

At 15^{th} day of ripening, total moulds on C1 cheeses was up to 2.8 x 10^3 cfu cm^{-2} (3.5 $\log_{10}$); subsequently, they gradually increased about 10 times reaching the value about of 10^6 cfu cm^{-2} (6.1 $\log_{10}$) at 100^{th} day.

In LCHE400 and LCHEP, moulds increased to 10^5 cfu cm^{-2} (4.9 $\log_{10}$) on the 40^{th} day of ripening and this value remained almost unchanged until to 80^{th} day. Total moulds detected on LCHE200 reached the maximum value at 100^{th} day of ripening while C2, LCHE400 and LCHEP showed the

lowest values, as exhibited in Table 1. A similar trend characterized the mould proliferation on C1 and LCHE200 during the whole period of ripening, reaching the highest value at 100^{th} days compared to the other points of sampling.

Considering the fungal species, the most abundant was *P. commune*, present in all cheeses regardless of treatment on the rind and the time of sampling. In contrast, *C. cladosporioides* proliferated only on C1, reaching the number of 4.2×10^4 cfu cm^{-2} (4.6 $\log_{10}$) and 5.2×10^5 cfu cm^{-2} (5.7 $\log_{10}$) respectively after 80 and 100 days of cheese ripening (Table 1).

The total number of yeasts on cheeses before treatments was 8.2×10^3 cfu cm^{-2} (3.1 $\log_{10}$). On C1 yeasts showed a fluctuating growth reaching the maximum value at 100^{th} day while on C2 remained constant at all points of sampling with the exception of a decrease at 40^{th} day. The lowest value has been found in LCHEP at 15^{th} day. Then, yeasts proliferated and the only differences were found respect to C1 at 40^{th} and 80^{th} day. At 100^{th} day, C2 and LCHE200 were similar but higher than LCHE400 and LCHEP. In any case the values detected on all cheeses were very low during the whole period of ripening (Table 2).

3.5 Proximate analysis, physical evolution and fatty acid profile.

The chemical composition of cheese and the oxidative status are reported in Table 3. No differences were found among cheeses with the exception of DM content that increased in LCHE400 and LCHEP. Moreover, the effect of treatments was not significant for fatty acid profile which was similar among cheeses during the ripening period. For the sake of simplicity, it has been not reported in Table 3.

206 CHE coloured the rind but not the cheese itself, stopping at the surface and not diffusing in the cheese.

207

208 **4. Discussion**

209 CHE treatment did not modify the chemical features of cheeses. Only CHE400 and the direct application of the powder (CHEP) seemed to increase
210 the loss of water during the ripening. The effect of treatment on the oxidative status of cheese was negligible in accordance with a very low presence
211 of oxygen inside the cheese matrix as consequence of a good ageing conditions (Zottola & Smith, 1993).

212 In all cheeses treated with CHE, the proliferation of *C. cladosporioides* did not occur, confirming the inhibition power of this extract previously
213 tested in the *in vitro* experiment and used on the rind either in solution or directly as powder. The findings is in accordance with polyphenolic nature
214 of CHE, in literature the antimicrobial activity of this compounds is well documented (Landete, 2011; Okuda, 2005; Tosi et al., 2013). Therefore, the
215 treatment of the cheeses with CHE was useful in preventing an aesthetic damages due to black spots which compromise the marketing of cheeses and
216 in the avoiding the potential production of mycotoxins, harmful to public health because this mould is able to grow at low oxygen level and to penetrate
217 into the texture (Cousin, 2003; Machado et al., 2012; Knutsen et al., 2012; Sharpe et al., 2015). Although the improved sanitation and control of dairy
218 plant air can contribute to a reduction in the level of mould spoilage of cheeses, *C. cladosporioides* is frequently found in several dairy products;
219 Jarlsberg or other Novegian cheeses (Kure, Skaar & Brendehaug, 2004), French cheeses such as St. Nectaire and Tome de Savoie (Gripon, 1993) or
220 even in cow milk and butter (Cousin, 2003; Delavenne et al., 2011); this mould being lipolytic, produces off-flavors including 2-methylisoborneol
221 and geosmin contributing to undesirable earthy flavour (Sørhaug, 2011).

222 The identification of mould proliferated on the cheese samples showed that *P. commune* was the only mould present on the rind of treated cheeses.
223 These data are in accordance with literature which showed that this species is able to grow on tannins (Lekha and Lonsane, 1997; Auguilar and
224 Gutierrez-Sanchez, 2001).

225 CHE 400 and CHEP were effective in inhibiting the proliferation of this mould within 15 days and in decreasing it in the last 20 days of ageing.
226 However, the proliferation of *P. commune* on cheeses represents a less serious aesthetic damage than that of *C. cladosporioides* because the mycelium
227 of this species can be easily removed from the surface of the cheeses by brushing before marketing. *P. commune*, in fact, frequently contaminates
228 foods and it was found on several cheeses such as Cheddar and on Italian goat's and sheep's milk cheeses (Hayaloglu & Kirbag, 2007; Hocking &
229 Faedo, 1992; Montagna et al., 2004; Kure et al., 2001). This microorganism is considered as the wild type ancestor of *P. camamberti* and is able to
230 grow in the air with high levels of carbon dioxide as that of long-ripening cheese chambers (Kure & Skaar, 2000). Several studies indicated that the
231 probability of detecting this mould follows a seasonal fluctuation, but the reason of this behaviour is not obvious (Kure et al., 2004; Ren, Jankun &
232 Leaderer, 1999). Air and cheese making equipment through the entire production line represent a crucial point of the potential contamination. In
233 contrast, even if in literature no current evidence for human toxicity is reported, *P. commune* could be potentially harmful for consumers because it
234 is able to produce cyclopiazonic acid that is a mycotoxin able to inhibit the Ca^{2+}-ATPase in the intracellular Ca^{2+} storage sites (Cousin, 2003; Sosa et
235 al., 2002). The impact of most non-starter moulds and yeasts in cheeses is not completely known and literature suggests that this microbiota could
236 induce a contribution to the flavour and texture in cheeses (Fleet, 1990; Pereira-Diaz, Potes, Marinho, Malfeito-Ferreira & Loureiro et al., 2000). CHE
237 and silver ion solutions used in this trial did not inhibit completely the mould and yeast proliferation, but their growth was affected by the kind of
238 solution and its molarity. However, the trend of microbial growth is very low. The presence of high numbers of *P. commune* on the samples treated

239 with silver ion solution is not easily explained because of its high ability in inhibiting the microbial colonisation in foods. It is possible that the
240 continuous use of this antimicrobial could have selected for *Penicillium* strains with high resistance to the silver ion polymer used. However, a lag-
241 time for the antimicrobial activities of polyphenols and ion solution against *P. commune*, within 80th day, could be considered. Moreover, the tannin
242 concentration used for the solution LCHE200 was not efficient to avoid microbial contamination by this microrganism, with a similar trend to the
243 untreated cheeses (C1). In contrast, silver ion (C2) and the other tanniferous treatments (LCHE400 and LCHEP) seem to be efficient after 80 days
244 from cheese making, decreasing the concentration of moulds on untreated or treated at 200g/L cheeses.

245 When the silver ion solution is used, the yeast proliferation was stable for the whole period of ripening with the exception of the sampling at 40th day,
246 maybe due of the intrinsic variability of samples. With CHE200, the growth of yeasts increased constantly even if the initial values were lower than
247 that of the other cheese units and the final values were comparable to those of C2. CHE400 and CHEP showed the yeasts growth only at 80 days but
248 it was very low.

249 In this trial, also a yeast static effect of CHE was observed during the first period of ripening (within 40 days). Usually, the cheese is contaminated by
250 an environment rich in yeasts when it is plunged in the brine. In particular, *D. hansenii*, is resistant to the salt solution (Butinar, Santos, Spencer-
251 Martins, Oren, & Gunde-Cimerman, 2005; Corte et al., 2006; Seiler & Busse, 1990) even if its presence, however, on the crust should not be considered
252 a problem. In fact, this species is an important component in the production of several cheese type made with ewes milk (Del Bove et al., 2009;
253 Cosentino, Fadda, Deplano, Mulargia & Palmas, 2001; Fadda, Mossa, Pisanu, Deplanu & Cosentino, 2004; Gardini et al., 2006). *D. hansenii* can
254 contribute to the development of the flavour exerting important metabolic activities during ripening and limiting the growth of deleterious spoiling
255 bacteria (Bonaiti, Leclercq-Perlat, Latrille & Corrieu, 2004; Cousin, 2003; Fatichenti, Bergere, Deiana & Farris, 1983). However, before marketing,

its cells can be easily removed from the surface of the cheese by washing or brushing. Compared to moulds, for yeasts the tanniferous treatments were effective at each point of sampling with the exception of 40th day according to a lag-time of efficiency. However, the mechanism of tannin action on microorganisms is not clear. The action can be multiple: breaking of cell membrane linking proteins, inhibition of enzymes, ion deprivation; however, for hydrolysable polyphenols, it is hypotisable also an indirect action by their metabolites after a first microbial metabolisation (Espin, et al., 2007; Patra & Saxena, 2011).

5. Conclusion

The results of the present study indicated that CHE is capable of inhibiting *C. cladosporioides* proliferation during the ripening of the semi-hard sheep cheese. The efficacy of CHE solutions to avoid the cheese spoilage started from 200 gL^{-1} and this concentration can be suggested as practical dose in cheese making procedure. An additional benefit of using a natural antimicrobial is the replacement of antibiotics or of synthetic chemical solutions whose use leads to problem of microbial resistance, of the environmental pollution, of food sanity for consumers, and of wastewater disposal.

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377

378 Figure 1. Loave with black spots formed by the fungal mycelium belonging to Cladosporium cladosporioides (A, top surface; B, lateral surface).

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1A 1B

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Figure 2. Electrophoretic pattern of rITS-PCR products from Cladosporium cladosporioides (Cc), Penicillium commune (Pc) and Debaryomyces hansenii (Dh). M indicates the DNA molecular weight marker.

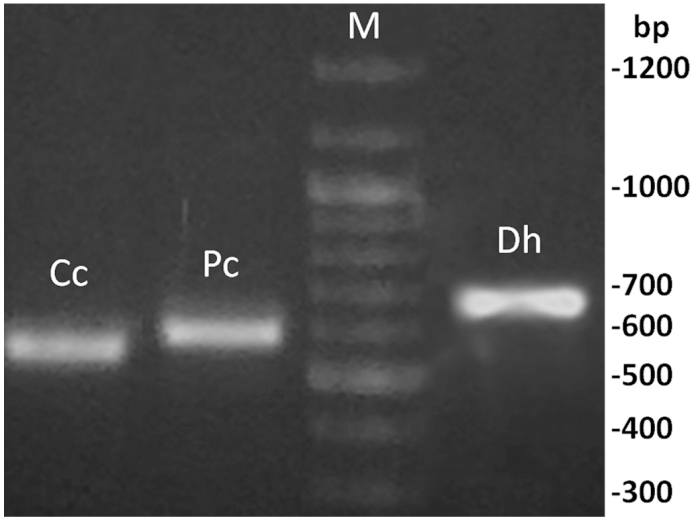

Figure 3. Presence of inhibition halo surrounding the diskette.

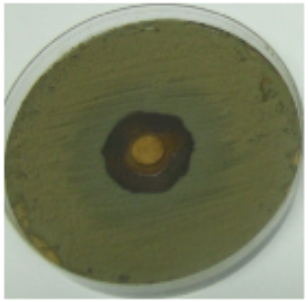

Table 1. Total moulds in cheese rind during the ripening period; the mean values are reported as $\log_{10}$ (CFU cm^{-2}), with their standard errors (SEM); (number of samples for each treatment at any time = 3).

Cheese treatment[1]	Time (days)					
	15	40	80	100	SEM	P
C1	$3.452^{b\delta 2}$	$4.270^{b\gamma}$	$^{*}5.110^{\beta}$	$^{*}6.072^{b\alpha}$	0.263	0.0001
C2	$5.107^{a\beta}$	$5.410^{a\alpha\beta}$	5.716^{α}	$4.974^{c\beta}$		
LCHE200	$4.841^{a\gamma}$	$5.299^{a\gamma\beta}$	5.601^{β}	$6.722^{a\alpha}$		
LCHE400	$2.185^{c\gamma}$	$5.390^{a\alpha}$	5.539^{α}	$4.708^{c\beta}$		
LCHEP	$2.945^{b\gamma}$	$5.237^{a\alpha}$	5.628^{α}	$4.542^{c\beta}$		

[1]**C1**, control 1 no treated; **C2**, control 2 treated with a commercial polymeric solution containing silver ion; **LCHE200**, cheese loaves treated with the solution of chestnut tannin extract at 200 gL^{-1}; **LCHE400**, cheese loaves treated with the solution of chestnut tannin extract at 400 gL^{-1};**LCHEP**, cheese loaves treated with the powder of chestnut tannin extract.

[2]Probability of significant effect due to the interaction between experimental factors Treatment and Time; α, β, γ, δ, ε within a row, means with different Greek superscripts are significantly different ($P<0.05$); a, b, c within a column, means with different Latin superscripts are significantly different ($P<0.05$).

* Development of *C. cladosporioides* with 32% and 45% of the total moulds, respectively after 80 and 100 days of cheese ripening.

Table 2. Total yeasts in cheese rind during the ripening period; the mean values are reported as $\log_{10}$ (CFU cm^{-2}), with their standard errors (SEM); (number of samples for each treatment at any time = 3).

Cheese treatment[1]	Time (days)			
	15	40	80	
C1	$4.525^{a\beta}$	2.698^{δ}	$3.288^{b\gamma}$	5
C2	$4.116^{a\alpha}$	3.454^{β}	$4.013^{a\alpha}$	4
LCHE200	$2.954^{b\beta}$	2.954^{β}	$3.954^{a\alpha}$	3
LCHE400	$2.920^{b\beta}$	2.954^{β}	$3.954^{a\alpha}$	2
LCHEP	$0.954^{c\gamma}$	3.013^{β}	$3.954^{a\alpha}$	3

[1]**C1**, control 1 no treated; **C2**, control 2 treated with a commercial polymeric solution containing silver ion; **LCHE200**, cheese loaves treated with the solution of chestnut tannin extract at 200 gL^{-1}; **LCHE400**, cheese loaves treated with the solution of chestnut tannin extract at 400 gL^{-1}; **LCHEP**, cheese loaves treated with the powder of chestnut tannin extract.

[2]Probability of significant effect due to the interaction between experimental factors Treatment and Time; α, β, γ, δ, ε within a row, means with different Greek superscripts are significantly different ($P<0.05$); a, b, c within a column, means with different Latin superscripts are significantly different ($P<0.05$).

421 Table 3. Chemical composition and oxidative status of control and treated cheeses at 100th day of
422 ripening.

items	[1]Treatments					[2]SEM	[3]P
	C1	C2	LCHE200	LCHE400	LCHEP		
DM mg kg^{-1} of cheese	750.5c	758.3c	793.6b	810.4a	812.6a	3.31	0.031
CP mg g^{-1} of DM	364.0	364.1	359.5	383.3	374.5	2.73	ns
Fat mg g^{-1} of DM	417.1	415.1	418.7	405.5	417.3	1.99	ns
TBARS μM	2.15	2.34	2.29	2.21	2.23	0.11	ns

423 [1]**C1**, control 1 no treated; **C2**, control 2 treated with a commercial polymeric solution containing
424 silver ion; **LCHE200**, cheese loaves treated with the solution of chestnut tannin extract at 200 gL^{-1};
425 **LCHE400**, cheese loaves treated with the solution of chestnut tannin extract at 400 gL^{-1};
426 **LCHEP**, cheese loaves treated with the powder of chestnut tannin extract.
427 [2] Mean values with their standard errors (SEM); number of samples for each treatment at any time = 3.
428 [3] Probability of significant effect due to the Treatment; a, b, c within the row, means with different Latin
429 superscripts are significantly different ($P<0.05$)
430