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Effect of storage on biochemical and microbiological parameters of edible truffle species

Roberta Saltarelli [a,*], Paola Ceccaroli [a], Paola Cesari [a], Elena Barbieri [b], Vilberto Stocchi [a,b,*]

[a] *Istituto di Chimica Biologica "Giorgio Fornaini", Università degli Studi di Urbino "Carlo Bo", Via A. Saffi, 2, 61029 Urbino (PU), Italy*
[b] *Istituto di Ricerca sull'Attività Motoria, Università degli Studi di Urbino "Carlo Bo", Via I Maggetti, 26, 61029 Urbino (PU), Italy*

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Abstract

The effects of different storage treatments on the most common edible truffle species, such as *Tuber magnatum* and *Tuber borchii* (white truffles), *Tuber melanosporum* and *Tuber aestivum* (black truffles), were analysed. Biochemical and microbiological profiles were monitored, in order to evaluate possible alterations during truffle preservation. After harvesting, some fresh samples were kept at 4 °C for 30 days, other samples were frozen at −20 °C for one month, thawed and preserved at 4 °C; the remainder were autoclaved.

The biochemical parameters studied were sugar and protein content, the activity of some enzymes involved in the central metabolism of the fungi and the electrophoretic pattern of soluble proteins. Total mesophilic bacteria were also counted. The results obtained showed that the storage at 4 °C is the treatment that best preserves the biochemical and microbiological characteristics of fresh truffles. Black truffles were more resistant to biochemical spoilage than the white ones, while *T. magnatum* was the most resistant to microbial spoilage.

Keywords: Bacteria; Enzymes; Food conservation; Proteins; Truffle

1. Introduction

Edible mycorrhizal mushrooms include some of the world's most expensive foods and have a global market measured in US$ billions (Hall, Yun, & Amicucci, 2003). In particular truffles, the fruit bodies of ascomycetous fungi belonging to the genus *Tuber*, are found worldwide and some of them have an important commercial value due to their particular aroma (Trappe, 1979).

Truffles are hypogeous fungi which live in symbiosis with host plant roots, in order to accomplish their complex life cycle (Harley & Smith, 1983). The life cycle involves a first phase of growth as filamentous mycelium, a second phase of symbiotic association of the fungal hyphae with the host root (ectomycorrhiza) and finally the organisation of a hypogeous fruit body (also called ascoma/ta, sporocarp/s or ascocarp/s) with asci and ascospores (Peterson & Bonfante, 1994). This cycle has been reported to be influenced by bacteria present in both the mycorrhizosphere (Duponnois & Garbaye, 1992) and fruit bodies of various truffle species (Barbieri et al., 2005). Several microbiological approaches to characterise the bacterial populations of truffle have been performed, clearly indicating that the bacterial community includes pseudomonads, aerobic spore-forming bacteria, actinomycetes and rhizobiaceae (Barbieri et al., 2007; Citterio et al., 1995; Sbrana et al., 2002).

Truffle species possess common ecological features, such as a wide range of host plant species and the need for a calcareous soil, but differ in their geographic distribution. *Tuber borchii* Vittad. and *Tuber aestivum* Vittad. are found throughout Europe (Riousset, Riousset, Chevalier, & Bardet, 2001), *Tuber melanosporum* Vittad. is collected in Italy, France and Spain, and *Tuber magnatum* Pico fruit bodies

* Corresponding authors. Address: Istituto di Chimica Biologica "Giorgio Fornaini", Università degli Studi di Urbino "Carlo Bo", Via A. Saffi, 2, 61029 Urbino (PU), Italy. Tel.: +39 0722 305262; fax: +39 0722 305324.
E-mail addresses: roberta.saltarelli@uniurb.it (R. Saltarelli), vilberto.stocchi@uniurb.it (V. Stocchi).

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have so far been collected in Italy and in Croatia, Slovenia and Hungary, in limited amounts (Mello, Murat, & Bonfante, 2006).

Among the different species *T. magnatum*, "white truffle", and *T. melanosporum*,"black truffle", are the most appreciated and their demand is increasing in the food markets of many countries. The culinary and commercial value of such edible fungi is mainly due to their organoleptic properties. For this reason, the volatile components of *Tuber* aroma have been extensively studied. Using headspace solid-phase microextraction (HS-SPME), combined with gas chromatography-mass spectrometry (GC/MS), it was possible to identify 40 compounds in *T. aestivum* and 70 compounds in *T. melanosporum* (Díaz, Ibáñez, Señoráns, & Reglero, 2003), 29 compounds in *T. borchii* (Gioacchini et al., 2005) and 36 compounds in *T. magnatum* (Bellesia, Pinetti, Bianchi, & Tirillini, 1996; Gioacchini et al., 2005). More recently, using stir-bar sorptive extraction (SBSE), coupled with GC/MS, a total of 119 volatile organic compounds were identified from the fruiting bodies of *T. melanosporum, T. indicum* and *T. borchii* (Splivallo, Bossi, Maffei, & Bonfante, 2007).

Although *Tuber* volatiles have been deeply investigated, in relation to both truffle species identification (Gioacchini et al., 2005; March, Richards, & Ryan, 2006) and to freshness evaluation (Falasconi et al., 2005), the knowledge of the physicochemical and microbiological modifications during truffle storage are still limited (Harki, Bouya, & Dargent, 2006; Nazzaro et al., 2007). Several methodologies to improve the shelf life of *Tuber* and to preserve its sensory and structural characteristics have been applied. For example, the utilisation of 1.5 kGy irradiation safeguards some biochemical characteristics and causes a decrease in the truffle microbial flora (Nazzaro et al., 2007). Nevertheless, for fresh or preserved truffle market, standard protocols exist regarding their preparation. Thus, fruit bodies, used as fresh products, are minimally processed and, to preserve quality and to extend the shelf life of the fungus, low temperature (4 °C) is often utilised, while truffles used for the production of a wide variety of food products such as oil, cheese, pasta, pizza, sauce and chocolate, are immediately frozen or sterilised by autoclave at 120–130 °C for 30 min.

Thus, the objective of this study was to determine changes in some biochemical parameters, such as enzymatic assay, protein pattern, and protein and sugar content, which occur during storage of fresh *Tuber* at 4–20 °C and after autoclave treatment. The extent of growth of naturally-occurring microrganisms was also determined under the conditions utilised in the storage.

2. Material and methods

2.1. Standards and reagents

Coenzymes, enzymes, substrates, β-mercaptoethanol (β-MSH) and dithiothreitol (DTT) were purchased from Sigma (St. Louis, MO), Bio-Rad protein assay dye reagent concentrate from Bio-Rad Laboratories (Hercules, CA), and plate counting agar (PCA) from Oxoid Ltd. (Basingstoke, UK).

2.2. Fungal material

Fresh samples of truffles were kindly supplied by Martelli Famiano's Company and belonged to the most important commercial species: *T. magnatum, T. borchii, T. aestivum* and *T. melanosporum*. The samples were harvested in Central Italy (Marche) over an entire harvesting season (2005–2006) specific for each species: *T. magnatum* from 1st October to 31st December, *T. borchii* from 15th to 30th April, *T. aestivum* from 1st May to 31st August and from 1st October to 31st December, and *T. melanosporum* from 1st December to 15th March. For each truffle species six samples were tested.

2.3. Truffle preparation

The fresh samples were washed with distilled water and the peridium was removed. All materials used for cutting and handling the fruit bodies were continually disinfected, and metallic surfaces were flamed at regular intervals. Each sample was cut with a sharp knife into small pieces (0.1–0.5 g), transferred into 15 ml sterile PP-Test tubes. Some tubes were stored at 4 °C for 0, 4, 8, 15 and 30 days. Other tubes were immediately dipped in liquid nitrogen and then stored at −20 °C; after one month of freezing the samples were transferred to a cold room, and then analysed after 0, 8, 24, 48, and 96 h at 4 °C. The last group of tubes were autoclaved and analysed at 0, 3 and 7 days, after storage at 4 °C.

2.4. Enzyme assay

The truffle pieces stored as described above were homogenised, using a Potter homogeniser with glass pestle (working capacity 2 ml: Steroglass, S.r.l., Perugia, Italy) in 5 mM KH_2PO_4/Na_2HPO_4 (pH 8.1) buffer containing 3 mM KF, 3 mM β-MSH, 1 mM DTT and 5 mM glucose (buffer A). The suspension obtained was then centrifuged at 14,000 rpm for 15 min. The supernatant was used for the activity assay of hexokinase (HK) (EC 2.7.1.1), phosphofructokinase (PFK) (EC 2.7.1.11), pyruvate kinase (PK) (EC 2.7.1.40), glucose-6-phosphate dehydrogenase (G6PD) (EC 1.1.1.49) and phosphoglucomutase (PGM) (EC 5.4.2.2), as described in Beutler (1984). One unit (U) of enzymes is defined as the amount of enzyme which catalyses the formation of 1 μmol of product/min at 37 °C. The values obtained were the means of six independent determinations.

2.5. Protein content

The soluble proteins were extracted with buffer A and the protein content was spectrophotometrically determined

at 595 nm using the Bio-Rad protein assay dye reagent concentrate, according to Bradford (1976). Bovine serum albumin was used as the standard reference.

2.6. Carbohydrate content

For the extraction of soluble carbohydrates, 0.5 g of dry weight of different truffle species were ground with a Potter homogeniser in liquid nitrogen. The monosaccharides were extracted with 12.5 ml methanol/water (80:20, v/v) at 4 °C. The fungal extract was then centrifuged at 3000 g for 15 min and the supernatants from two successive extractions in methanol/water were pooled and lyophilised at 65 °C. For the polysaccharide determination, the remaining pellet was extracted with hot water ($T = 100$ °C, 3 h), and precipitated with 96% ethanol. The mono- and polysaccharide content for each species was determined by anthrone colorimetric method (Wang et al., 2002).

2.7. SDS–PAGE

The truffle pieces stored as described above were homogenised using a Potter homogeniser in buffer A containing 0.5% (v/v) Triton X-100. SDS–PAGE was carried out using vertical slab gels (16 cm × 18 cm × 1 mm) under denaturing conditions, according to Laemmli (1970). Polyacrylamide gels (12%) running under a constant voltage of 60 V for stacking and of 180 V for separating were used. Low and high molecular weight proteins (Bio-Rad) were used as standards and the gels were stained with Coomassie Brilliant Blue R-250.

2.8. Microbial analysis

Ascocarps were washed with sterile water and gently brushed; the surfaces of the ascocarps were briefly flamed and about 1 g of sample was removed from the inner part of each ascocarp and homogenised for 1 min in 10 ml of sterile physiological solution (NaCl 0.85%). Further decimal dilutions were made and then 0.1 ml of each dilution was pipetted onto the surface of PCA. The plates were then incubated for 72 h at 30 °C for mesophilic bacteria count, as reported in Ercolini, Russo, Torrieri, Masi, and Villani (2006). Colony forming units (CFUs)/gram of ascocarps, subjected to different storage conditions were calculated.

2.9. Statistical analysis

Six samples of each *Tuber* species were individually analysed and the results are shown as means ± SD ($n = 6$). The evaluation of statistical significance was determined by a one-way analysis of variance and multiple comparison analyses were performed by the Kruskal Wallis test. p-values less than 0.05 were considered significant.

3. Results and discussion

The effects of storage on the biochemical constituents of different *Tuber* species were investigated.

3.1. Protein and sugar content

In Table 1 is shown the total soluble protein content of truffles from *T. magnatum*, *T. borchii*, *T. melanosporum* and *T. aestivum* species. The ascomata, ready for the market, possessed a difference in maturation degree (50–80% of asci containing mature spores), which did not affect the total protein content.

The comparison of total protein revealed that, among the four species analysed, the white truffle *T. magnatum* possessed a higher protein content than the other ones. Few papers have studied the chemical composition and nutritional values of different *Tuber* species (Coli, Maurizi Coli, Granetti, & Damiani, 1988). From a nutritional point

Table 1
Soluble protein content in ascomata from different *Tuber* species during storage

Ascomata	Storage at 4 °C	*T. magnatum*	*T. borchii*	*T. melanosporum*	*T. aestivum*
Fresh	0 day	24.0 ± 1.02^{a}	13.1 ± 1.26^{a}	8.7 ± 0.83^{a}	11.0 ± 0.93^{a}
	4 days	25.0 ± 0.86^{a}	11.8 ± 0.67^{a}	8.3 ± 0.93^{a}	11.9 ± 0.36^{a}
	8 days	21.1 ± 0.76^{a}	12.5 ± 1.35^{a}	8.7 ± 0.68^{a}	10.8 ± 1.27^{a}
	15 days	21.5 ± 0.56^{a}	10.8 ± 1.83^{a}	8.7 ± 0.54^{a}	12.6 ± 1.23^{a}
	30 days	20.5 ± 1.20^{b}	5.6 ± 0.66^{b}	7.5 ± 0.43^{a}	12.9 ± 1.05^{a}
Frozen/thawed	0 h	12.5 ± 0.82^{a}	11.7 ± 1.23^{a}	7.2 ± 0.75^{a}	9.4 ± 0.75^{a}
	8 h	11.5 ± 0.92^{a}	$8.5 \pm 0.89^{b,c,d}$	$6.7 \pm 0.68^{b,c}$	$8.7 \pm 0.98^{b,c}$
	24 h	11.6 ± 0.63^{a}	$8.4 \pm 0.75^{c,d}$	$5.4 \pm 0.87^{c,d}$	9.9 ± 1.23^{a}
	48 h	7.8 ± 0.76^{b}	7.7 ± 0.39^{d}	4.5 ± 0.35^{d}	7.5 ± 0.85^{c}
	96 h	7.5 ± 0.56^{b}	6.7 ± 0.45^{e}	2.5 ± 0.45^{e}	4.4 ± 0.32^{d}
Autoclaved	0 day	6.9 ± 0.32^{a}	2.7 ± 0.72^{a}	1.5 ± 0.09^{a}	1.0 ± 0.65^{a}
	3 days	3.8 ± 0.52^{b}	1.4 ± 0.15^{b}	1.2 ± 0.14^{b}	1.6 ± 0.23^{b}
	7 days	2.2 ± 0.11^{b}	1.9 ± 0.23^{b}	1.0 ± 0.05^{b}	0.9 ± 0.03^{a}

The values are expressed as g/100 g of ascomata dry weight. Means with different letters within the same group (species/storage condition) are significantly different ($p < 0.05$).

of view the proteins are important because they are complete, rich in cysteine, methionine and lysine and possess good digestibility. Furthermore, the enzymatic ultrafiltrate comparable digest amino acid index (EUD) has allowed evaluation of the *Tuber* protein quality, which is comparable to that found in legumes, such as beans, peas and lentils.

Significant differences in proteins were observed due to the different storage conditions. In particular, at 4 °C the fresh *T. magnatum*, *T. melanosporum* and *T. aestivum* samples were not greatly affected in their total protein content, whereas a large decrease in the *T. borchii* species occurred mainly at the end of storage (30 days). The samples, immediately frozen at −20 °C and then kept at 4 °C for several hours, had different behaviour. *T. magnatum* showed a rapid decrease in protein content immediately after thawing (48%), but the value remained almost constant during the entire storage period (a decrease of 66% at 96 h). On the other hand, the other three species showed a small decrease immediately after thawing (10–18%), but after 96 h the protein content showed a rapidly decrease, reaching, for example, about 72% in *T. melanosporum*. The autoclave treatment resulted in a significant lost of total protein content (∼90%) in all species tested.

Table 2 shows the soluble carbohydrate content of the four fresh *Tuber* species. The data reported for *T. magnatum* and *T. melanosporum* are comparable to those present in the literature (Coli et al., 1988), whereas no information is available for *T. borchii* and *T. aestivum*. The soluble carbohydrates were at a very low percentage compared to proteins and, among the four truffle species, *T. aestivum* possessed the highest content of carbohydrates, mainly as soluble polysaccharides. The sugar content was not affected by the conservation treatments (data not shown) but the carbohydrate content varied with the different maturation stage of the harvested truffles. In fact, it has been already reported that *T. melanosporum*, analysed at three different stages of maturation, showed variation in its chemical, which involved mainly its carbohydrate content (Harki et al., 2006). This variation can be explained by the fact that in immature truffles the mycelial structure is abundant, whereas in the mature fungi, spore structures are preponderant and rich in sugars. These free carbohydrates, such as mannitol, seem to have a reserve function for the spores (Jennings, 1995).

3.2. Electrophoretic analyses

When soluble proteins were fractionated by SDS–PAGE, the profiles showed an evident and expected variability among different *Tuber* species; some bands may be considered as species-specific (Fig. 1). In fact, predominant electrophoretic bands were typical for each truffle species and there was no correspondence, even between the patterns of morphologically very similar species (for example *T. magnatum* and *T. borchii*). In the *T. magnatum* SDS profile there was a major and specific band around 31.0 kDa. In this species the protein profile did not change during storage of the fresh fruit bodies at 4 °C, while the pattern of frozen samples at −20 °C and then kept at 4 °C lost some bands between 21.5 and 31.0 kDa. Fresh *T. borchii* kept at 4 °C for 30 days showed changes in its electrophoretic profile: a band of about 45 kDa disappeared and two bands at about 25 and 20 kDa increased in intensity. During storage at 4 °C, fresh *T. melanosporum* fruit bodies showed similar electrophoretic profiles, although a band around 31.0 kDa progressively decreased from 8 days of the storage period. The same band did not seem to be affected by the freezing process, remaining constant even after defrosting the samples. However, the intensity of some spots between 14.4 and 31.0 kDa of frozen ascomata decreased during the conservation time at 4 °C. *T. aestivum* possessed a protein pattern with a higher number of bands compared with the other species. Numerous spots reduced in size and intensity during 4 °C storage, both in fresh and frozen samples, and, in particular, a band between 45 and 31.0 kDa decreased proportionally during the time that the frozen sample was maintained at 4 °C. It has been reported that the truffle proteins are quite susceptible to degradation even during storage at −20 °C, and the best results were obtained adding specific inhibitors of proteolytic activities to the sample immediately after the extraction (Lazzari, Gianazza, & Viotti, 1995). The electrophoretic profiles confirmed that freezing causes the degradation of some proteins.

As shown also in Fig. 1, the fruit bodies appeared to be completely degraded after the autoclave treatment. The truffle proteins were completely degraded, as were, probably, other components of important nutritional value. *T. melanosporum* and *T. magnatum*, which are the two truffle species most appreciated, are rich in proteins, in minerals

Table 2
Carbohydrate content of *Tuber* species

	T. magnatum		*T. borchii*		*T. melanosporum*		*T. aestivum*	
	Range	Mean	Range	Mean	Range	Mean	Range	Mean
Mono	0.51–0.58	0.53[a]	0.35–0.42	0.38[a]	0.15–0.32	0.25[b]	0.41–0.48	0.42[a]
Poly	1.35–2.10	1.70[a]	2.41–4.12	3.21[a]	1.31–1.75	1.52[a]	4.01–7.13	5.23[b]
Total		2.23		3.59		1.77		5.65

Mono, monosaccharides; Poly, polysaccharides. The data are given both as average and minimum–maximum range due to the different maturation degree of truffles. The values are expressed as g/100 g of ascomata dry weight. Means with different letters within the same line are significantly different ($p < 0.05$).

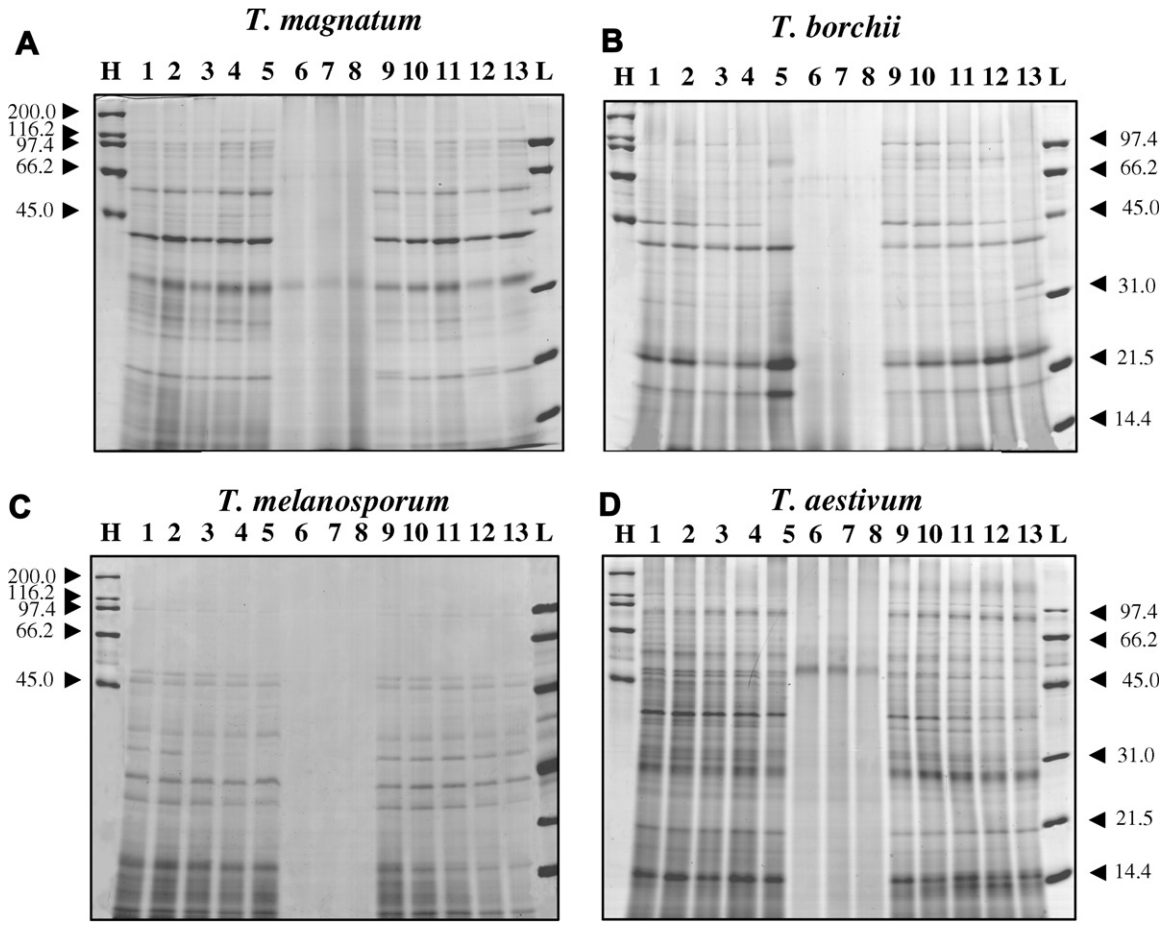

Fig. 1. SDS–12% PAGE of crude extract from different *Tuber* species. (A) *Tuber magnatum*; (B) *Tuber borchii*; (C) *Tuber melanosporum*; (D) *Tuber aestivum*. Fifty micrograms of total proteins per line were loaded onto the gels. H, high molecular weight; line 1, control sample (0 days); lines 2–5, fresh fruit bodies conserved at 4 °C for 4, 8, 15 and 30 days, respectively; line 6, fruit bodies autoclaved immediately; lines 7–8, fruit bodies autoclaved and then kept at 4 °C for 3 and 7 days; lines 9–13, fruit bodies frozen for one month at −20 °C and then kept at 4 °C for 0, 8, 24, 48 and 96 h, respectively; L, low molecular weight.

(potassium, phosphorus, iron and calcium), in sulphur-containing amino acids, and in fatty acids, such as the essential linoleic acid (Harki et al., 2006).

3.3. Enzymatic determination

The activity levels of some enzymes were evaluated in the four *Tuber* species during the different conservation methods. In Table 3 are reported the values for hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK), which represent the three irreversible steps specific for glycolysis; glucose-6-phosphate dehydrogenase (G6PD), an enzyme involved in the pentose phosphate pathway, which supplies reducing energy to cells by maintaining the level of NADPH; and phosphoglucomutase (PGM), which is a key enzyme in the metabolic pathway for galactose biosynthesis. Among the three glycolytic enzymes assayed, HK did not decrease when the fruit bodies were stored at 4 °C or frozen at −20 °C. PFK maintained constant activity until 15 days, whereas freezing caused a decrease, which was more evident in *T. aestivum*. The PK enzyme was quite constant but in *T. aestivum* it showed a slow inactivation during storage at 4 °C, both in fresh and in frozen/thawed samples. Glycolysis is a nearly universal pathway for energy generation in living cells. The energy requirements during a cellular life cycle are variable and energy production should be well coordinated at all times. Cells have developed a complex energy sensory mechanism assembled around the behaviour of PFK, which catalyses the phosphorylation of fructose 6-phosphate to fructose 1,6-biphosphate, in the presence of magnesium and adenosine triphosphate. This reaction represents an irreversible step, specific for glycolysis. In the four *Tuber* species examined, PFK was the most sensitive to storage treatments, in comparison with HK and PK, indicating that the lost of enzymatic activity should be due to proteolytic processes occurring during storage or to the effect of allosteric inhibitors. In fact, it is known that among all the glycolytic enzymes, PFK is controlled by many allosteric effectors (activators and inhibitors), evidencing the key role that the enzyme plays in the regulation of the glycolytic flux (Hofmann, 1976; Ruiz, Kopperschläger, & Radermacher,

Table 3
Levels of some enzymes in fresh or frozen/thawed ascomata from different *Tuber* species

Fresh truffles						Frozen/thawed truffles (−20 °C)					
Storage 4 °C (days)	HK	PFK	PK	G6PD	PGM	Storage 4 °C (h)	HK	PFK	PK	G6PD	PGM
Tuber magnatum											
0	0.12[a]	0.19[a]	0.37[a]	0.14[a]	1.21[a]	0	0.15[a]	0.24[a]	0.20[a]	0.05[a]	0.87[a]
4	0.13[a]	0.21[a]	0.47[a]	0.18[a]	1.43[a]	8	0.16[a]	0.23[a]	0.20[a]	0.03[a]	0.98[a]
8	0.10[a]	0.19[a]	0.38[a]	0.19[a]	1.12[a]	24	0.18[a]	0.24[a]	0.35[a]	0.03[a]	0.73[a]
15	0.13[a]	0.28[a]	0.42[a]	0.20[a]	0.75[b]	48	0.18[a]	0.12[b]	0.34[a]	0.05[a]	0.80[a]
30	0.14[a]	0.12[b]	0.39[a]	0.16[a]	0.50[b,c]	96	0.21[a]	0.12[b]	0.32[a]	0.04[a]	0.43[b]
Tuber borchii											
0	0.12[a]	0.10[a]	0.29[a]	0.03[a]	1.11[a]	0	0.15[a]	0.11[a]	0.25[a]	0.03[a]	0.93[a]
4	0.09[a]	0.07[b]	0.29[a]	0.03[a]	0.87[a]	8	0.12[a]	0.06[b]	0.21[a]	0.04[a]	0.71[b]
8	0.09[a]	0.08[b]	0.34[a]	0.02[a]	0.87[a]	24	0.14[a]	0.06[b]	0.22[a]	0.01[a]	0.36[c]
15	0.10[a]	0.07[b]	0.27[a]	0.01[a]	0.71[b]	48	0.14[a]	0.05[b]	0.21[a]	0.006[b]	0.14[d]
30	0.13[a]	0.07[b]	0.23[a]	n.d.	0.18[c]	96	0.16[a]	0.06[b]	0.20[a]	n.d.	0.06[e]
Tuber melanosporum											
0	0.17[a]	0.57[a]	0.24[a]	0.25[a]	0.82[a]	0	0.17[a]	0.53[a]	0.20[a]	0.20 [a]	1.10[a]
4	0.21[a]	0.62[a]	0.29[a]	0.31[a]	0.81[a]	8	0.21[a]	0.52[a]	0.19[a]	0.25 [a]	1.10[a]
8	0.20[a]	0.61[a]	0.33[a]	0.28[a]	0.82[a]	24	0.15[a]	0.17[b]	0.14[b]	0.33 [a]	0.85[b]
15	0.22[a]	0.51[a]	0.31[a]	0.32[a]	1.21[a]	48	0.15[a]	0.04[c]	0.12[b]	0.20 [a]	0.56[c]
30	0.16[a]	0.36[b]	0.25[a]	0.28[a]	1.17[a]	96	0.20[a]	0.02[c]	0.12[b]	0.20 [a]	0.38[d]
Tuber aestivum											
0	0.13[a]	0.28[a]	0.76[a]	0.14[a]	4.06[a]	0	0.11[a]	0.06[a]	0.63[a]	0.11[a]	4.08[a]
4	0.17[a]	0.30[a]	0.66[a]	0.12[a]	4.19[a]	8	0.13[a]	0.08[a]	0.32[b]	0.17[a]	2.96[b]
8	0.14[a]	0.19[b]	0.60[a]	0.17[a]	3.43[b]	24	0.11[a]	0.04[a]	0.17[c]	0.14[a]	2.48[b]
15	0.13[a]	0.24[a]	0.54[b]	0.19[a]	3.42[b]	48	0.10[a]	n.d.	0.15[c]	0.10[a]	1.58[c]
30	0.14[a]	0.12[b]	0.41[b]	0.12[a]	3.21[b]	96	0.15[a]	n.d.	0.14[c]	0.12[a]	1.08[c]

Enzymatic activities are expressed as Unit/mg of total proteins. Each value represents the mean of six independent measurements and varied from the mean by not more than 5%. Means with different letters within the same column are significantly different ($p < 0.05$).

2001). G6PD did not decrease during storage at 4 °C of fresh sample, whereas it was strongly inhibited in the *T. borchii* and *T. magnatum* frozen samples. Furthermore, this enzyme showed a very low activity in *T. borchii* fruit body, as reported in our previous paper, concerning a preliminary study on the biochemical characterisation of various species of white truffles (Cardoni et al., 1995).

PGM was the only enzyme which significantly decreased its activity. In fact, in *T. magnatum* and *T. borchii* the enzyme decay ranged from 50% to 90%, both in fresh and frozen samples. Only in fresh *T. melanosporum* and *T. aestivum* PGM did not change significantly during storage at 4 °C, but it decreased by about 65% and 75% in the frozen fruit bodies kept at 4 °C for 96 h, respectively. Cytosolic PGM plays a central role in carbohydrate metabolism and its decay should correlate with the physiological development and structural modifications, which occur during the ageing of ascocarps. In literature some putative genes differentially expressed during the development from a completely immature (0% of asci with mature spores) to mature ascomata (76–100% of asci with mature spores) have been identified (Gabella et al., 2005; Zeppa et al., 2002). So far, studies at the molecular level concerning the progressive ageing of mature ascomata are missing and this enzyme could represent a biochemical marker for truffle senescence. In fact, we have previously demonstrated that this enzyme decreases also in mycelium when its growth level reaches a plateau even when the nutritional sources in the growth medium were not significantly affected (Saltarelli et al., 1999).

3.4. Microbial profile

Previous studies have been performed on the natural microbial communities of different truffle species. In *T. borchii* the presence of alpha-, beta-, gamma-proteobacteria, aerobic spore-forming bacteria and actinomycetes was described (Barbieri et al., 2005); in *T. aestivum* the microbial profile was mainly represented by lactobacilli, lactococci, micrococcaceae, clostridia, mesophilic bacteria and enterobacteriaceae (Nazzaro et al., 2007); a microbial community composition similar to the one described in *T. borchii* was also found in *T. magnatum*, independently from the ascocarp maturation degree (Barbieri et al., 2007). The total mesophilic bacteria in fresh collected truffle species showed a range of 10^7–10^8 CFU/g (Barbieri et al., 2005, 2007; Nazzaro et al., 2007). Only for *T. aestivum* it has been reported that the microbial count at time zero and after 30 days at 4 °C of storage was constant, except for enterococci content, which increased about fourfold (Nazzaro et al., 2007). The bacterial strains found in truffles have been described as root-associated bacteria and some of them show bivalent interactions with plant and

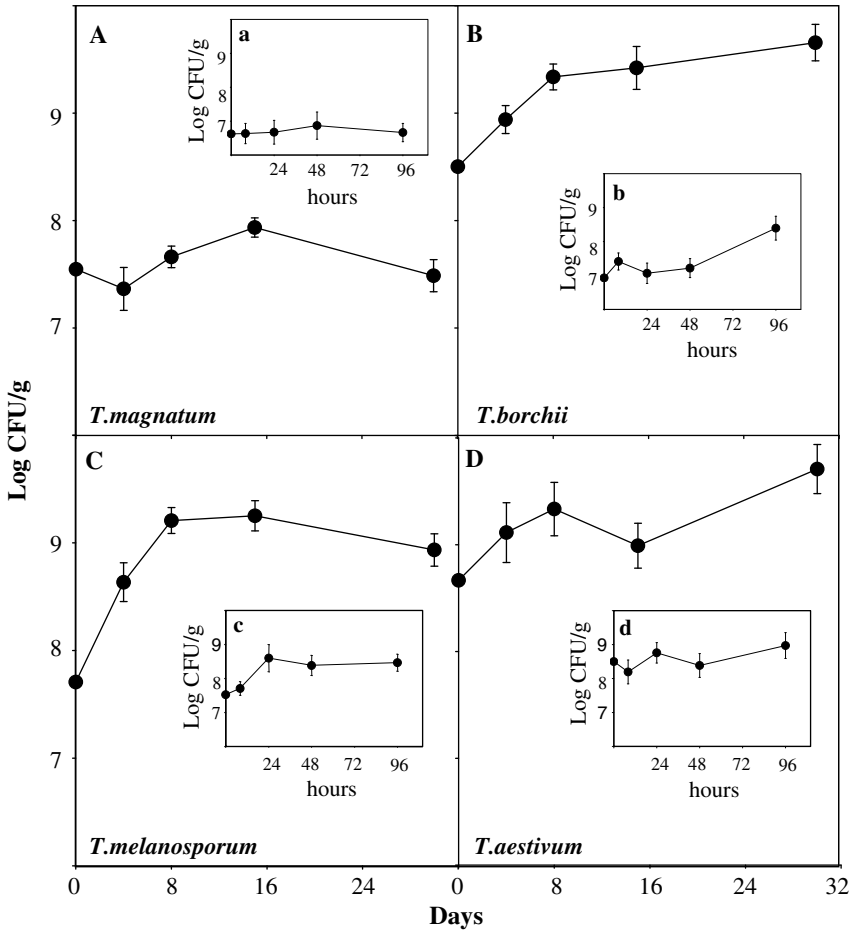

Fig. 2. Microbial growth during storage at 4 °C of fresh and frozen/thawed (insets) *Tuber* species. A–D fresh fruit bodies stored at 4 °C and analysed at 0, 4, 8, 15 and 30 days; a–d: fruit bodies frozen at −20 °C, then kept at 4 °C and successively analysed at 0, 8, 24, 48 and 96 h. Values are the mean of six independent counts for each species analysed.

human hosts (Berg, Eberl, & Hartmann, 2005). How much an ascocarp represents a reservoir for opportunistic human pathogens still remains unclear. Since truffles are mainly consumed uncooked, it is necessary to monitor the microbial abundance of fresh truffles, without altering their organoleptic properties, in order to guarantee the safety of the consumers.

Plate count analysis of different *Tuber* species was performed in fresh and frozen ascocarps for the determination of total mesophilic bacteria (Fig. 2). Several authors have demonstrated the potential use of the presence and amount of mesophilic microflora, for determining temperature abuse in foods and for predicting shelf life (Ercolini et al., 2006; Liu, Yang, & Li, 2006). This analysis is important for truffles, since they represent a particular microbial ecosystem and the associated microflora enhance truffle degradation, reducing its shelf life. As shown in Fig. 2, among the four *Tuber* spp. analysed, only *T. magnatum* (Fig. 2A) presented a microbial community which did not reveal large differences in the number of CFU/g during the conservation processes. On the contrary, fresh *T. borchii*, *T. melanosporum* and *T. aestivum* (Fig. 2B–D) showed a rapid increase in microbial population within 4–8 days, reaching a plateau of about 10^{10} CFU/g after 15 days of 4 °C storage. During the storage of minimally-processed cantaloupe melon, an increase in microbial population occurred within 24 h of storage at 20 °C, whereas at 4 °C there was an induction period of about 5 days before a rapid bacterial growth occurred (Lamikanra, Chen, Banks, & Hunter, 2000).

The analysis of the samples frozen and then kept at 4 °C (Fig. 2A–D insets) showed a reduction in the size of the truffle's bacterial community of about one order of magnitude in the CFU/g. *T. borchii* showed an increasing number of CFU/g after 48 h at 4 °C, indicating a probable predominance of psychrophilic species.

4. Conclusion

The effects of storage temperature, time, freezing and autoclaving method on biochemical and microbiological characteristics of four *Tuber* species were statistically significant. It is readily apparent that the conservation of fresh fruit bodies at 4 °C represents the most efficient method for

the preservation of truffles. The assessments revealed that, until 30 days, the biochemical modifications were acceptable without affecting the quality of ascomata much. Furthermore, the data obtained revealed that there are significantly differences among the four *Tuber* species examined during the different treatments. In particular the white truffles, *T. borchii* and *T. magnatum*, are more sensitive to biochemical spoilage than the black ones, *T. melanosporum* and *T. aestivum*. Regarding the microbial profile, *T. magnatum* is the most resistant to spoilage. These results are very important because the edible truffle species, well-known as white and black truffles, besides being highly regarded by chefs and gourmets due to the flavours they impart to foods, are in great demand in world trade.

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