

Sanitizing Method Effects on Depending-Culture Microorganisms in *Tuber aestivium*

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Abstract

Tuber aestivum/uncinatum has been widely used as food, food additives, and traditional medicine. Truffles are extremely perishable with a short postharvest life quality requiring a special handling for marketing in order to delay its deterioration. This study aimed to assess the effects of different sanitizing methods on superficial *Tuber aestivium* quality ascocarp. The results showed that the best treatment was obtained by immersing the Truffle ascocarps in boiling water for 1 or 2 min where counts of total mesophilic microorganisms (TMM) were respectively 81 and 7 CFU per g of dry Truffle ascocarps biomass, respectively. However, the highest TMM was obtained after rinsing Truffle ascocarps in 2% NaOH where recovery was 10^8 CFU per g of dry Truffle ascocarps biomass. Treatments applied to disinfect Truffle ascocarps were classified by increasing degree of efficiency as follows to reduce the microbial load expressed in CFU/g: Dipping in boiling water (2 min) 7 ± 3.41 ; dipping in boiling water (1 min) 81 ± 25.8 , rinsing with alcohol $2.10^2 \pm 13$; rinsing with tap water $6.10^3 \pm 36$; rinsing with H_2O_2 $6.10^4 \pm 2$; brushing $2.10^5 \pm 28$ and rinsing with NaOH $10^8 \pm 15$.

Keywords

Tuber aestivium Ascocarps, Total Count Mesophilic Bacteria, Molds and Yeast Decontamination

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1. Introduction

Truffles are the most highly edible valued mycorrhizae fungi in gastronomic and economic terms. Some species of Truffle such as *Tuber magnatum*, *Tuber melanosporum* and *Tuber aestivum/uncinatum* are highly praised due to their aroma and organoleptic properties [1]. Truffles are extremely perishable with a short postharvest shelf life which requires special handling for marketing in order to delay its deterioration. The deterioration of Truffle organoleptic quality is caused by the mold mycelia growth on the surface of Truffle and loss of water, which affects their visual appearance and flavours. *Tirmeria* and *Terfezia* species are usually eaten cooked whereas *Tuber* species are sometimes eaten uncooked.

Several studies reported high microbial loads in the range of 10^5 cfu/g to 10^8 cfu/g in the gleba of *Tuber borchii*, *Tuber magnatum*, *T. melanosporum* and *T. aestivum* species [2]-[4]. High microbial loads and the presence of potential pathogens such as *Listeria* and *Salmonella* species make Truffle spoilage easier [5]. It is essential for developing disinfection techniques to extend Truffle storage and to improve sanitary quality.

Early decontamination treatment can reduce the initial microbial loads thus increases the storage period. Several techniques have been used to reduce microorganisms on whole and cut fresh fruit, and their effectiveness depends on the product type, mitigation protocol, and other parameters. Contamination prevention seems to be the best way to eliminate pathogens from products even this is not always possible. The necessity of washing and sanitizing remains of paramount importance to prevent product spoiling outbreaks. It is well known that washing and sanitizing methods can't totally eliminate pathogens. Therefore, sanitizing methods are usually used to reduce the total flora existing. Some berries are sensitive to washing due to their susceptibility to mold proliferation. So similar fruit are often packaged in the field with a light post harvest handling and washing.

The increase in demand for *T. aestivum* leads food technologists to develop appropriate methods for its long and short term storage in order to retain its quality and reduce the threat of scarcity. After harvesting, *T. aestivum* is rapidly altered at room temperature and at 4°C due to natural flora existing in the Truffle ascocarps and to water content (76%). Moreover, Truffle ascocarps are subterranean fruit bodies growing underground, and it is well known that soil serves as a natural habitat for various microbial communities growing along with ascocarps [6]. It has been shown that bacteria can promote the establishment of ectomycorrhizal symbiosis [7]-[9].

Several researchers analyzed the microbial load in different Truffle species. These microbial communities step in growing, physiology and preservation of Truffle ascocarps [10] [11]. The microbial consortium could have an additive or antagonistic effect on Truffle preservation.

Different decontamination methods such as ionizing radiation, ultrasound, electron beam radiation, gamma rays and refrigeration at 4°C have been applied to minimize the microbial load of fresh Truffle [11] [12]. However, storage at a lower temperature is not an absolutely reliable method since many molds are able to grow at low temperatures. High concentration of chlorine (500 ppm) has been tried for decontamination but with unsatisfactory results due to high content of organic matter in Truffle and production of potential carcinogens such as trehalo-methanes [13]. Chemical fungicides have also been applied for preventing fungal decay; however, consumers demand minimum use of chemical preservatives. As a biological control, Lactic acid bacteria (LAB) have been reported to produce organic acids, bacteriocins, hydrogen peroxide, and low molecular weight antimicrobial agents which act as an anti-fungal agent [14]. Sorrentino *et al.* (2013) [15] reported that strains of *Lactobacillus plantarum* can be successfully used to prevent the growth of molds belonging to penicillium genus during storage of black Truffle at refrigeration temperature.

The goal of this study is to find an appropriate decontamination method that will reduce the microbial load of fresh Truffle in order to increase its shelf-life.

2. Material and Methods

2.1. *T. aestivum* Ascocarps Samples

The ascocarps of *T. aestivum* were collected in the experimental Truffle field of Juva Truffle Center (JTC), Juva, Finland, with the help of hunter Truffle dogs. The field soil was sandy with pH 7.2. The main host trees of *T. aestivum* were *Quercus peduncularis*, *Quercus robur* L and *Corylus avellana* L. To avoid cross contamination, the Truffle ascocarps were individually wrapped in plastic bags and transported to the laboratory in an insulated boxes for microbial analysis. The number of Truffle ascocarps used for this study was about 11 weighing 20 - 25 grams.

2.2. Soil Sample

The adhered soil to the Truffle ascocarps is considered as a separate sample to enumerate the natural associated flora and it was obtained by ascocarps brushing under laminar flow.

2.3. Quantitative Microbiological Analysis

Eight different decontamination treatments were applied to Truffle ascocarps: Brushing pending wholly extraction of adhered surface ascocarps soil, rinsing with municipal drinking water (preliminary analysis showed that the tap water is exempt of bacteria and was used this water to be near the habits of Finish people when they clean the Truffle by brush or by water), rinsing with ethanol [70%], dipping in boiled water during one or two min, rinsing with NaOH [2%] and rinsing with H₂O₂ [30%].

2.4. Truffle Ascocarp and Extract Preparation

Bacteria colonize both the external (peridium) and internal part (gleba) of Truffle [16]. For this, we not only considered the surface ascocarps bacterial extraction but also the total ascocarp body. Briefly, in a laminar hood and under sterile conditions, each piece of Truffle ascocarp was treated and then cut into 2 - 4-mm length pieces. Ten g treated Truffle samples with moisture content of 76% (dry weight) was suspended in 90 ml of sterile saline solution [0.9% w/v NaCl]. The mixture was submitted to a mechanical agitation for 1 hour at 240 rpm to allow bacteria extraction [17].

2.5. Enumeration of Bacteria

Enumeration of viable aerobic heterotrophic bacteria was performed by standard plate count procedures, in Plate Count Agar (PCA), Malt Extract Agar (MEA) and Yeast Extract Agar (YEA) (Difco, Germany). Plates were incubated in Biolog incubator at 25°C, and the number of Colony Forming Units (CFU) were determined after 48 h and the average number of microorganisms per gram of each sample was then calculated and reported as g of dried Truffle ascocarp or soil. Plate Count Agar medium were adjusted to a final pH = 7 ± 0.2. This medium is specific for total count bacteria. Malt Extract Agar medium Sigma-Aldrich 70145 appropriate for molds and yeast present in gram per liter of purified water Malt extract 30.0; Mycological peptone 5.0 Agar 15.0 final pH= 3.5 +/- 0.2 was adjusted by 10% of citric acid instead of 5% of tartaric acid.

The typical formula of Yeast Extract Agar medium Oxoid BO0635M per liter of distilled water included 3 g of Yeast Extract, 5 g of peptone and 15 g of Agar, final pH adjusted to 7 ± 0.4. This medium is suitable for both *P. aeruginosa* and *E. coli*.

2.6. Statistical Analysis

Results related to each treatment were repeated three times and analyzed by the SPSS statistical program (SPSS for Windows, SPSS Inc.). Values mentioned in **Table 1** are the average of three replicates and means were separated by the least significant difference according to the Student-Newman-Keuls Test. Pearson correlation determinations were performed using SPSS statistical analysis.

3. Results and Discussion

3.1. Microbial Enumeration in *T. aestivum* Ascocarps

The results showed that the more effective treatment was obtained when Truffle ascocarps were immersed in boiling water for 1 or 2 min with a total mesophilic microorganisms (TMM) count respectively 81 and 7 CFU per g of dry Truffle ascocarps biomass. However, the highest TMM was obtained after rinsing Truffle ascocarps in 2% NaOH where the number saved was 10⁸ CFU per g of dry Truffle ascocarps biomass. Applied treatments were classified with degree efficiency expressed in CFU/g: Dipping in boiled water (2 min) 7 ± 3.41; dipping in boiled water (1min) 81 ± 25.8, rinsing with ethanol (70%) 2. 10² ± 13; rinsing with tap water 6.10³ ± 36; rinsing with H₂O₂ 6 10⁴ ± 20, brushing 2. 10⁵ ± 28; rinsing with NaOH 10⁸ ± 15. The same order of classification was obtained for total count bacteria, molds-yeast and *P. aeruginosa-E. coli*. However, 10⁸ of TMM number present after NaOH and H₂O₂ was relatively high. In fact, freshly collected Truffles are associated with significant mi-

crobial populations. Studies assessing the total microbial load of entire ascocarps obtained different results. Nazzaro *et al.*, (2007) [10] detected a total microbial load in ascocarps of *T. aestivium* near to 10^7 CFU/g. However, Reale *et al.*, (2009) [11] reported only 4.0 CFU/g. These differences may be due to different procedures used to remove ascocarps surrounding soil. The development of superficial mold growth is one of the main problems in the postharvest phase of ascocarps affecting their visual quality, aroma and taste [18].

The significant difference in CFU number per gram of ascocarps between treatments generates four main groups. The first group included boiling treatments for 1 or 2 min. rinsing with ethanol treatment represents the second group, the third group contains rinsing with tap water and brushing; treatments with H_2O_2 and NaOH constitute the last group.

Results showed in **Table 1** reveal that the microbial population in *T. aestivium* is responsive to the type of treatment applied. This suggests that the treatment application may induce the number of microorganisms extracted. In fact, actively growing bacteria were effectively expressed by the decontamination method. The highest number of micro flora was obtained after NaOH or H_2O_2 treatment. This result presumed that NaOH or H_2O_2 induced release of microorganisms and positively affected extraction the yield of bacteria. Based on H_2O_2 effect, only bacteria producing catalase may survive in presence of H_2O_2 [19]. The catalyzed H_2O_2 molecule may provide oxygen gas to the medium as indicated in **Figure 1**. This reaction may also induce the aerobic bacteria development. The whole and high number of bacteria 10^8 CFU/g per dry matter expressed following H_2O_2 treatment may suggest the presence of mainly aerobic bacteria the catalase enzyme is absent in nearly all anaerobic bacteria and may thereby be suppressed by H_2O_2 . It is well known that some bacteria are able to produce H_2O_2 and could be used as a substrate for its growing. In fact, related to work of Eschenbach *et al.*, (1989) [20],

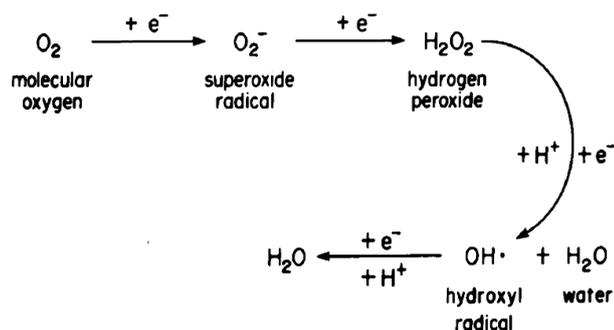


Figure 1. Intermediates in the Univalent Pathway of Oxygen Reduction (Archbald and Fridovich; 1983) [34].

Table 1. Microbial load expressed in CFU/g per dry matter of *T. aestivium* ascocarps in different applied treatments and in its adhered soil.

Treatments	PCA (1)	MEA (2)	YEA (3)
Brushing	$2.10^5 \pm 28$ (a)	$9.10^3 \pm 71$ (a)	$2.10^4 \pm 74$ (a)
Rinsing with tap water	$6.10^3 \pm 36$ (b)	$8.10^3 \pm 11$ (a)	$2.10^3 \pm 26$ (b)
Rinsing with ethanol (70%)	$2.10^2 \pm 13$ (c)	$2.10^2 \pm 40$ (b)	$5.10^2 \pm 11$ (c)
Dipping in boiled water (1 min)	81 ± 25.8 (d)	3 ± 1.8 (d)	$10^2 \pm 17$ (c)
Dipping in boiled water (2 min)	7 ± 3.41 (d)	1.4 ± 0.73 (d)	1.2 ± 2 (d)
Rinsing with NaOH	$10^8 \pm 15$ (e)	$6.10^7 \pm 23$ (e)	$9.10^7 \pm 19$ (e)
Rinsing with H_2O_2	$6.10^4 \pm 20$ (f)	$10^8 \pm 44$ (f)	$5.10^7 \pm 19$ (e)
Adhered soil	$2.10^9 \pm 16$ (g)	$5.10^6 \pm 27$ (g)	$10^7 \pm 10$ (e)

(1) Plate Count Agar, (2) Malt Extract Agar, (3) Yeast Extract Agar. Ethanol [70%, v/v], dipping in boiled water during one and two minutes, rinsing with H_2O_2 [30%, v/v]. (a, b, c, d, e, f, g) means number followed by the same number were not significantly different at $p = 0.05$ comparison established between lines using SPSS statistical program (SPSS for Windows, SPSS Inc.). Values mentioned are the average of three replicates.

some *Lactobacillus* are known to produce hydrogen peroxide inducing an increase in the number of *Lactobacillus*.

Decontamination method using boiled water during one minute keeps 2Ulog of *P. aeruginosa* and *E. coli* higher than total bacteria and fungi (molds and yeast) number. It is well known that *E. coli* and other pathogens can be removed by a simple pasteurization. The presence of this high load of *E. coli* may be related to a highly resistant natural flora of the ascocarps. In fact, during its life cycle, *T. aestivium* generates a radial stress zone producing volatile substances able to affect the bacteria community around the ascocarps and likely induce bacteria resistance to diverse stresses [21] [22].

The induced alkaline environment is another type of ascocarps stress. In fact, when ascocarps were cleaned with 2% NaOH, total bacteria, fungi and *P. aeruginosa*-*E. coli* count increased. The large number of microorganisms achieved after NaOH application can be a result of specific flora extraction with a shift of the initial pH of 5 of Truffle ascocarps to neutral pH. Balanced pH may promote the development of some bacteria and inhibition of fungi growing [23]. Unlike, another study showed that various high pH cleaners containing sodium hydroxide, potassium hydroxide, sodium bicarbonate, and/or sodium orthophenylphenate (with or without surfactants) reduced populations of *E. coli* on orange surfaces [24]. The same study revealed that high pH waxes used on fresh market citrus provided substantial inactivation of *E. coli* on the surfaces oranges fruit [25]. The high pH of typical alkaline wash solutions and concerns about environmental discharge of phosphates may be limiting factors for use of certain alkaline compounds on produce. Furthermore, Somers *et al.*, (1994) [26] in a laboratory study of suspended and attached cells of various foodborne pathogens on non-food surfaces showed that *E. coli* O157:H7 populations were reduced by 5 and 6 CFU after a 30-s decontamination method with 1% tri-sodium phosphate. Besides, Hwang and Beuchat, (1995) [27] working in chicken skin disinfection revealed that 0.05% NaOH significantly reduced the *Salmonella* spp. population but had no effect on *L. monocytogenes*. The difference in effectiveness degree to remove bacteria with alkaline solution may be the result of many parameters interaction such as pH, water content in Truffle ascocarp and its own composition (hydrocarbons, proteins, fatty acids contents).

Likewise, in the present study, ethanol may be considered as a potential disinfectant of Truffle ascocarps since the number of total microflora decreased 3 CFU compared to brushing. It is well known that ethanol is used in many fields such as clinical and food, it possess a large specter effect on bacteria discharge. Ethanol has pleiotropic effects on bacteria, membrane damage and inhibition of macromolecular biosynthesis being the major mechanisms. However, many bacteria may escaped the ethanol effect when the load of bacteria is very important or when the bacteria is not present at surface or covered by organic matter. Zhang *et al.* (2009) [28] suggested dipping in 80% ethanol for 10 seconds combined with mercuric chloride as the most effective surface disinfection method for inactivating *Escherichia coli* O157:H7 on lettuce leaves and roots.

As reported by Rivera *et al.*, (2011b) [6], after gamma irradiation treatments, two species of yeasts survived and became the dominant microbial populations; this selective effect of disinfectant and resistance of one group rather others suggest the necessity to combine treatment in order to enhance decontamination. For example, Rivera *et al.*, (2011b) [6] showed that 70% ethanol combined with ultrasound has a greater efficacy to reduce microbial load.

3.2. Microbial Presence in the Adhered Soil of *T. aestivium*

The layer of soil obtained after brushing Truffle ascocarps may be considered as the source of specific flora present in Truffle ascocarps. Wherefore, the adhered soil microflora of *Tuber. aestivium* ascocarps has been highlighted in the present work.

The number of CFU per gram of the adhered soil was respectively about 2.8×10^8 CFU/g of TMM bacteria, 4.7×10^6 CFU/g of both mold and yeast and about 1.8×10^6 CFU/g of both *P. aeruginosa* and *E. coli*. The high number of microflora in the adhered soil of *T. aestivium* substantiates the result of Rougieux (1963) [29]. Results showed that desert truffle can excrete substances able to stimulate the development of these microorganisms. The abundance of TMM bacteria, mold, yeast, *P. aeruginosa* and *E. coli* has been located in *T. borchii* soil [4]. This study showed obviously that TMM bacteria were about 10^2 CFU/g dry matter higher than fungi or both *E. coli* and *P. aeruginosa*. A similar study carried out by Dib-Bellahouel and Fortas (2014) [30] showed an estimated total count about 8.3 to 8.6×10^7 CFU/g of dry soil, while fungal genera present only 8.6×10^4 to 4.5×10^5 CFU/g. Thus, it is possible to suggest that the number of bacteria and fungi in adhered soil is balanced by

the Truffle presence, this suggestion need to be more demonstrated in other studies.

The soil microflora of Truffle ascocarps has been severally studied; most of them characterized types and abundance of bacteria such as Actinomycetes, yeasts and filamentous fungi [3] [31] [32]. Although, specific bacterial communities of *Tuber* species were analyzed, a core micro-biome composed of α -Proteobacteria from the family of Bradyrhizobiaceae seems to be common to all *Tuber* species studied [3] [4] [16].

Moreover, many studies have been conducted on bacterial diversity soil horizon containing Truffle. The horizon 0 - 20 cm depth showed the highest microbial diversity [33]. These authors enumerated the number of microorganisms respectively in soil adhered to *Terfezia boudieri* ascocarps and in 0 - 10 cm depth horizon, thus it was demonstrated that some microbial communities were higher in soil adjacent to *Terfezia boudieri* ascocarps than the horizon of 10 cm depth.

Further analysis need to advice that an ideal disinfection treatment should be simple and easy to use; it will be efficient in Truffle for its sensory characteristics preservation such as, aroma, texture and taste. Rivera *et al.*, (2011) [6] showed that in the last week of storage, the aroma of non-decontaminated *T. aestivum* samples was modified and associated to a strange odor of fungi. This loss of typical aroma influences the overall acceptability of the non-decontaminated Truffle out of marketability on day 28. The typical aroma odor and quality in the decontaminated summer Truffle samples moderately decreased in the first week of storage.

Chemical disinfectants and chemical residues, depending on their concentrations render Truffles to be unfit for human consumption and pose a threat for human health.

4. Conclusion

Microbial populations of Truffle were very complex and heterogeneous and were mostly mesophilic bacteria. Dipping Truffle ascocarps in boiled water for 1 - 2 minutes reduced microbial load and seems to be an efficient method recommended before *T. aestivum* storage.

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