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Microbial Safety of Black Summer Truffle Collected from Sicily and Umbria Regions, Italy

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HIGHLIGHTS

- High microbial loads were detected in fresh ascomata of black summer truffles collected from Central Italy.
- *Pseudomonas* spp. and lactic acid bacteria were present at the highest levels.
- Common brushing procedure applied for preparation of truffles is not sufficient to eliminate microbial risks.

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Acronyms and abbreviations
CFU=Colony Forming Unit
CNS=Coagulase-Negative Staphylococci
CPS=Coagulase-Positive Staphylococci
LAB=Lactic Acid Bacteria
TMC=Total Mesophilic Count
TPC=Total Psychrotrophic Count

ABSTRACT

Background: *Tuber aestivum* Vittad., known as black summer truffle, represents high-value food especially used as garnishment in nouvelle cuisine. The aim of this study was to investigate on the viable microbial populations associated with *T. aestivum* ascomata collected in different sites of Sicily and one locality of Umbria (Italy).

Methods: The ripe ascomata of black summer truffles were collected from Central Italy. Cell densities of spoilage bacteria, fecal indicators, potential pathogens, yeasts, and molds were analyzed. Statistical analysis was conducted with XLSTAT software.

Results: The microbiological counts of truffles ranged between 6.00 and 9.63 log Colony Forming Unit (CFU)/g for total mesophilic count and between 6.18 and 8.55 log CFU/g for total psychrotrophic count; pseudomonads were in the range 6.98-9.28 log CFU/g. *Listeria* spp. and coagulase-positive streptococci detected in no samples. Coagulase-negative streptococci were found in some samples with 2.11-4.76 log CFU/g levels. Yeasts and filamentous fungi were detected at consistent levels of 3.60-7.81 log CFU/g. Significant differences (p<0.01) were found between samples and also for all microbial groups.

Conclusion: This study evidenced that the common brushing procedure applied for preparation of truffles is not sufficient to eliminate microbial risks for consumers. The application of an efficient decontamination treatment is strongly suggested before consumption of fresh truffles.

Introduction

Truffles are symbiotic fungi which grow in association with the roots of broad-leaved and conifer trees in ectomycorrhizal associations. This type of associations, prevalent in boreal and temperate forests, has high relevance in ecosystem because improves nutrition and health of plant (Buscot et al., 2000; Martin et al., 2001; Read, 1991). Truffles are essential for agronomy and forestry and the ascomata of some *Tuber* species, being

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edible, are more and more requested for their organoleptic properties (Zambonelli et al., 2014).

The most common truffle species collected in Italy is *Tuber aestivum*, known as black summer truffles or "scorzone" (Calvo et al., 2020; Comi et al., 2010, Venturella et al., 2011). Truffles ectomycorrhizas and fruiting bodies harbor a different microbial community including bacteria, yeasts, and filamentous fungi (Barbieri et al., 2005, 2007; Buzzini et al., 2005; Pacioni et al., 2007). Similarly to vegetables, the contamination of truffles depends on several factors, especially wild animals (including truffle dogs during ascomata collection), air, and especially soil (Buck et al., 2003). Indeed, soil hosts many bacterial populations, including human pathogen species that might be transferred to truffles representing the most critical point for the safety of these products.

The aim of this study was to investigate on the viable microbial populations associated with *T. aestivum* ascomata collected in different sites of Sicily and one locality of Umbria (Italy) which are generally sold in local markets without any decontamination procedure. For this reason, the classical culture-dependent microbiological approach was applied.

Materials and methods

Samples

The ripe ascomata of black summer truffles (*T. aestivum*) were collected between June and November 2019 in different forest areas of Sicily (n=7) and a locality of Umbria (n=1) with the support of a trained dog. Truffles were collected with their covering soil and transported under refrigerated conditions by a thermal insulated box containing reusable ice packs to the Laboratory of Agricultural Microbiology, Department of Agricultural, Food and Forestry Sciences (SAAF) of the University of Palermo for analyses. Truffles weight ranged between 10 and 35 g, the shape was almost spherical with a diameter 4-6 cm, the external surface was dark and covered by pyramidal warts; the internal pulp was compact and aromatic. Truffle samples were named with the code "TAR" followed by a progressive number according to reception order; specifically TAR 1, TAR 4, TAR 5, TAR 7, and TAR 8 came from different site of Burgio (AG), TAR 2 from Castelbuono (PA), and TAR 9 from Lucca Sicula (AG), all located in Sicily region, and TAR 10 from Perugia (PG), the sole sample from Umbria region. Before microbiological analyses, all samples were brushed with a stainless steel mushroom brush (Ernesto, OWIM GmbH & Co. KG, Neckarsulm, Germany) with soft plastic bristles to avoid damages during soil removing. An example of the truffle samples ready to be analyzed is reported in Figure 1. In order to avoid cross-contamination among samples, the brushing procedure was performed under a laminar flow hood and the brush was washed, rinsed with ethanol and subjected to 30 min UV treatment before brushing the truffles of the different sites.

Microbiological analysis

Each sample (10 g) were aseptically collected and transferred into sterile BagLight® 400 Multilayer® bags (Interscience, Saint Nom, France), made in PolySilk®. Ringer's solution (Sigma-Aldrich, Milan, Italy) was added to reach a 10:1 dilution and the truffle samples were homogenized by the stomacher BagMixer® 400 lab blender (Interscience, Saint Nom, France) at the maximum speed for 2 min.

Truffle samples were serially diluted (10:1 ratio) with Ringer's solution (Sigma-Aldrich, Milan, Italy) and investigated for the following 14 microbial groups including Total Psychrotrophic Count (TPC), Total Mesophilic Count (TMC); alterative microorganisms such as enterococci, pseudomonads, Coagulase-Negative Staphylococci (CNS), coccus and rods Lactic Acid Bacteria (LAB); pathogens such as *Listeria* spp. and enteric Gram-negative microorganism; alterative or potentially pathogens such as coliforms, members of the Enterobacteriaceae family, clostridia, Coagulase-Positive Staphylococci (CPS); and eukaryotic microorganism such as yeasts and moulds.

Cell suspensions were plated and incubated as follows: TPC and TMC were spread plated on Plate Count Agar (PCA) and incubated aerobically at 22 °C for 7 days and 30 °C for 72 h, respectively; total coliforms were pour plated on Violet Red Bile Agar (VRBA), incubated aerobically at 37 °C for 24 h; members of the Enterobacteriaceae family were pour plated on Violet Red Bile Glucose Agar (VRBGA), incubated aerobically at 37 °C for 24 h; *Pseudomonas* species on *Pseudomonas* Agar Base (PAB) supplemented with *Pseudomonas* Cephaloridine-Fucidin-Cetrimide (CFC), incubated aerobically at 20 °C for 48 h; enterococci on Kanamycin Aesculin Azide (KAA) agar, incubated aerobically at 37 °C for 24 h; clostridia were first heated at 80 °C for 30 min in order to destroy the vegetative cells and then plated on reinforced clostridial agar, incubated anaerobically at 37 °C for 48 h; members of *Listeria* genus on Fraser agar with specific supplement, incubated aerobically at 37 °C for 24 h; enteric Gram-negative on Hektoen Enteric Agar (HEA), incubated aerobically at 37 °C for 24 h; CPS and CNS on Baird Parker (BP) agar with rabbit plasma fibrinogen supplement, incubated aerobically at 37 °C for 24 h; coccus LAB on M17 agar

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with cycloheximide (10 mg/ml) to avoid fungal growth, incubated anaerobically at 30 °C for 48 h; rod LAB on de Man-Rogosa-Sharpe (MRS) agar with cycloheximide, incubated anaerobically at 30 °C for 48 h; yeasts on Yeast extract Peptone Dextrose (YPD) agar, incubated aerobically at 25 °C for 48 h and moulds on Malt Agar (MA), incubated aerobically at 25 °C for 7 days. To inhibit bacterial growth, chloramphenicol (0.05 mg/ml) was added to YPD and MA; on the contrary, cycloheximide (10 mg/ml) was added to MRS and M17 to avoid fungal growth.

All media and supplements were purchased from Oxoid (Milan, Italy). Microbiological tests were carried out in triplicate for each sample. Plate counts were determined as Colony Forming Unit (CFU)/g and converted to $\log_{10}$ (log) values. The results were expressed as mean±Standard Deviation (SD).

Statistical analyses

Statistical analyses were carried out with XLSTAT software version 7.5.2 for Excel (Addinsoft Inc., New York, NY, USA). Means differences were calculated by Tukey's multiple-range test. Differences were considered significant at $p<0.05$.

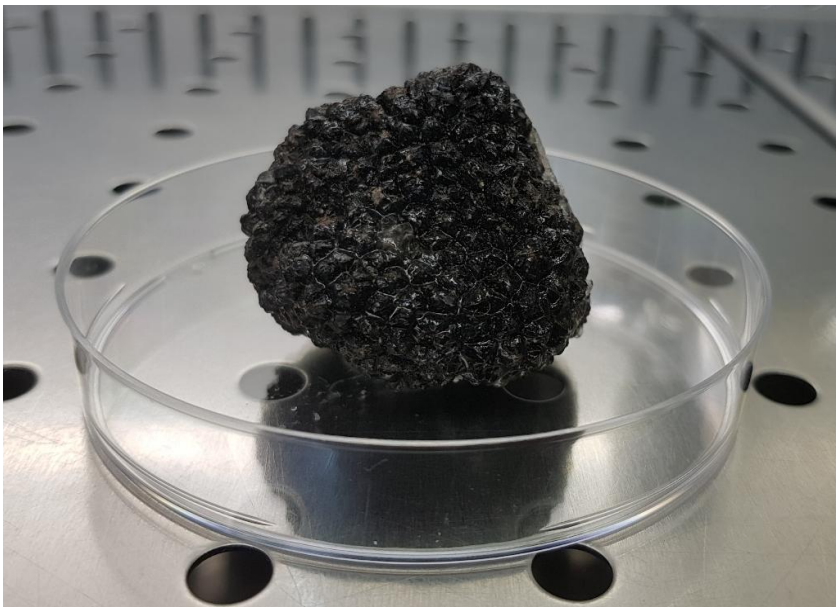

Figure 1: A truffle sample after brushing

Results

The truffle samples of the different sites showed different microbial loads for all investigated microbial groups. Results of total and spoilage microorganism counts were shown in Table 1; and potentially pathogenic and eukaryotic microorganism counts were illustrated in Tables 2 and 3, respectively. For all samples the highest cell densities were estimated on PCA incubated at 30 °C; TMC ranged between 6.00 and 9.63 log CFU/g. However, also TPC were particularly high for almost all samples with loads between 6.18 and 8.55 log CFU/g. Surprisingly, the sample TAR 9 showed TPC levels below the detection limit.

Pseudomonads were in the range 6.98-9.28 log CFU/g and, also in this case, the sample TAR 9 resulted below the detection limit. Regarding LABs, cocci were found at higher levels than rods. Members of the Enterobacteriaceae family and, within this, total coliforms were detected in all samples at levels ranging from 4.00 to 7.00 log CFU/g. Enterococci were present in all truffles with values between 3.00 and 5.15 log CFU/g.

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Clostridia did not exceed 4.50 log CFU/g in the majority of samples, but were not detected in the samples TAR 2 and TAR 8. *Listeria* spp. was not detected in any samples. CPS neither were detected in any samples, but CNS were found in some samples (TAR 5, TAR 7, and TAR 9) with levels in the range 2.11 and 4.76 log CFU/g. Enteric Gram-negative count showed three different morphologies on Hektoen Enteric Agar medium: red colonies indicating the presence of presumptive *Escherichia coli* in all samples; green colonies imputable to the presence of presumptive *Shigella* spp. detected in almost all samples, except TAR 7 and TAR 8; blue-green colonies with black centre showing the potential presence of *Salmonella* spp. only in samples TAR 10 at 3.18 log CFU/g.

Regarding the eukaryotic organisms, yeasts were detected at consistent levels (4.40-7.81 log CFU/g) in most of the samples, but were not detected in samples TAR 1 and TAR 4, while filamentous fungi were found in all samples except TAR 2 and TAR 10 at levels 3.60-4.65 log CFU/g. Significant differences (p<0.01) were found between samples and also for all microbial groups.

Table 1: Total and spoilage microorganism counts of truffle samples

Samples	Microbial loads (log CFU/g)						
	TMC	TPC	*Pseudomonas* spp.	Coccus LAB	Rod LAB	Enterococci	CNS
TAR 1	8.95±0.21 ^{b}	6.93±0.23 ^{c}	8.90±0.25 ^{ab}	8.70±0.28 ^{ab}	5.81±0.25 ^{b}	4.98±0.28 ^{a}	<2.00 ^{d}
TAR 2	8.30±0.18 ^{c}	6.85±0.25 ^{c}	8.40±0.28 ^{b}	8.60±0.15 ^{abc}	5.18±0.28 ^{c}	3.00±0.15 ^{b}	<2.00 ^{d}
TAR 4	7.30±0.23 ^{d}	6.18±0.28 ^{d}	6.98±0.31 ^{d}	7.27±0.23 ^{d}	4.77±0.28 ^{cde}	4.77±0.13 ^{a}	<2.00 ^{d}
TAR 5	8.03±0.11 ^{c}	7.44±0.25 ^{bc}	7.59±0.22 ^{c}	8.30±0.11 ^{bc}	4.30±0.22 ^{de}	3.18±0.23 ^{b}	4.76±0.33 ^{a}
TAR 7	9.45±0.15 ^{ab}	8.02±0.27 ^{ab}	8.30±0.13 ^{b}	8.85±0.15 ^{ab}	4.88±0.13 ^{cd}	3.00±0.22 ^{b}	4.32±0.15 ^{a}
TAR 8	9.63±0.25 ^{a}	8.55±0.19 ^{a}	9.28±0.12 ^{a}	9.20±0.25 ^{a}	6.79±0.12 ^{a}	3.40±0.22 ^{b}	<2.00 ^{d}
TAR 9	6.00±0.28 ^{e}	<2.00 ^{e}	<2.00 ^{e}	5.70±0.25 ^{e}	3.70±0.19 ^{f}	3.40±0.28 ^{b}	3.70±0.33 ^{b}
TAR 10	9.06±0.33 ^{ab}	7.00±0.28 ^{c}	7.41±0.23 ^{cd}	8.06±0.28 ^{c}	4,18±0.15 ^{ef}	5.15±0.28 ^{a}	2.11±0.09 ^{c}
Statistical significance	***	***	***	***	***	***	***

-TMC: Total Mesophilic Count; TPC: Total Psychrotrophic Count; LAB: Lactic Acid Bacteria; CNS: Coagulase Negative Staphylococci
-Results indicate mean values±SD of three plate counts (carried out in triplicate). Data within column followed by the same letter significantly different based on Tukey's test.
-*: p≤0.05; **: p≤0.01; ***:p≤0.001; N.S.: Not Significant

Table 2: Potentially pathogen microorganism loads of truffle samples

Samples	Microbial loads (log CFU/g)					
	Clostridia	Total coliforms	Enterobacteriaceae	*Escherichia coli*	*Shigella* spp.	*Salmonella* spp.
TAR 1	3.54±0.27 ^{cd}	4.77±0.19 ^{cd}	6.13±0.15 ^{b}	4.60±0.19 ^{cd}	4.81±0.28 ^{a}	<2.00 ^{d}
TAR 2	<1.00 ^{e}	5.16±0.23 ^{bc}	6.89±0.11 ^{a}	4.69±0.23 ^{bc}	4.85±0.15 ^{a}	<2.00 ^{d}
TAR 4	3.00±0.15 ^{d}	4.39±0.28 ^{de}	4.82±0.25 ^{c}	4.44±0.28 ^{cd}	4.00±0.18 ^{b}	<2.00 ^{d}
TAR 5	3.65±0.18 ^{bc}	4.00±0.25 ^{e}	6.04±0.19 ^{b}	4.43±0.33 ^{cd}	4.24±0.25 ^{b}	<2.00 ^{d}
TAR 7	4.50±0.25 ^{a}	4.27±0.28 ^{de}	6.06±0.28 ^{b}	5.29±0.19 ^{b}	<2.00 ^{d}	<2.00 ^{d}
TAR 8	<1.00 ^{e}	5.81±0.11 ^{ab}	7.00±0.22 ^{a}	7.48±0.19 ^{a}	<2.00 ^{d}	<2.00 ^{d}
TAR 9	3.30±0.23 ^{cd}	5.54±0.28 ^{ab}	4.54±0.15 ^{c}	3.00±0.15 ^{e}	2.70±0.25 ^{c}	<2.00 ^{d}
TAR 10	4.11±0.28 ^{ab}	6.10±0.31 ^{a}	6.29±0.23 ^{b}	4.00±0.25 ^{d}	4.88±0.13 ^{a}	3.18±0.28 ^{a}
Statistical significance	***	***	***	***	***	***

-Results indicate mean values±SD of three plate counts (carried out in triplicate). Data within column followed by the same letter significantly different based on Tukey's test.
-*: p≤0.05; **: p≤0.01; ***:p≤0.001; N.S.: Not Significant

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Table 3: Eukaryotic microorganism loads of truffle samples

Samples	Microbial loads (log CFU/g)	
	Yeasts	Molds
TAR 1	<2.00 [d]	3.70±0.23 [b]
TAR 2	4.60±0.19 [c]	<2.00 [d]
TAR 4	<2.00 [d]	3.70±0.28 [b]
TAR 5	6.99±0.23 [b]	4.65±0.25 [a]
TAR 7	7.81±0.28 [a]	4.54±0.23 [a]
TAR 8	7.10±0.15 [b]	4.18±0.11 [ab]
TAR 9	4.40±0.28 [c]	3.70±0.15 [b]
TAR 10	6.90±0.25 [b]	<2.00 [d]
Statistical significance	***	***

-Results indicate mean values±SD of three plate counts (carried out in triplicate). Data within column followed by the same letter significantly different based on Tukey's test.
-*: $p \leq 0.05$; **: $p \leq 0.01$; ***:$p \leq 0.001$; N.S.: Not Significant

Discussion

The following work is the consequence of the hypothesis that truffles, because their development takes place inside soil and their consumption is mainly raw, can be subject to high bacterial contamination, also in terms of potentially pathogenic bacteria for humans as reported in other previous work (Nazzaro et al., 2007; Rivera et al., 2011; Sorrentino et al., 2018).

Results of microbiological analysis indicated TMC at dominant levels up to 9.63 log CFU/g, followed by pseudomonads and coccus LAB up to 9.28 log CFU/g. These results indicated that the main populations of truffles are coccus LAB and pseudomonads, typical spoilage bacteria of several foods of vegetable origin (Francesca et al., 2019; Miceli et al., 2019). Similar TMC levels were reported by other authors on truffle ascomata collected in Molise region, Italy, and Aragon region, Spain (Comi et al., 2010; Rivera et al., 2010; Sorrentino et al., 2013, 2018). In particular, Comi et al. (2010) reported TMC loads of 9.50 log CFU/g in *T. aestivum* samples collected in Molise region, comparable to the loads found for TAR 1, TAR 7, TAR 8, and TAR 10. Rivera et al. (2010) besides whole truffle, also investigated on the microbial loads of gleba and peridium; as expected, the highest mean loads (8.50 log CFU/g) of microorganisms were revealed on the external part of truffles (peridum), almost superimposable to the levels registered for samples TAR 2 and TAR 5. Other works analyzed microbiological parameters of several mushrooms and shown similar results of TMC in fresh wild or cultivated mushrooms (Venturini et al., 2011) and in minimally processed sliced mushrooms (Jiang et al., 2018; Kim et al., 2016).

The presence of high levels of mesophilic microorganisms can be imputable to the high temperatures registered during summer period in Italy, when *T. aestivum* harvest occurs (Rivera et al., 2010). High temperatures are also favorable to LAB development and this might explain the high load of LAB cocci detected in all truffle samples analyzed in our work. The cell densities of TPC were lower than those recorded for TMC in all samples. No previous works on the microbiological characteristics of truffles focused on the evaluation of the levels of TPC. However, TPC values detected in the present work followed the general trend observed for wild and cultivated mushrooms. Gaglio et al. (2019) showed TPC loads in range of 6.85-7.86 log CFU/g in wild mushrooms, comparable results were also showed by Jiang et al. (2018) who found mean load of 7.80 log CFU/g in sliced cultivated mushrooms.

The levels of pseudomonads estimated in the present work are very similar to those recorded by Rivera et al. (2010) on whole *T. aestivum* (6.1-9.0 log CFU/g). Some authors suggested that pseudomonads could have positive action in the truffle life cycle (Bedini et al., 1999; Sbrana et al., 2002), but, after harvesting, they basically act as spoilage microorganisms, especially for their psychrotrophic characteristics since they are able to multiply at the low temperatures applied during storage. Venturini et al. (2011) also showed that pseudomonads could reach more than 90% of total counts in mushroom samples. Furthermore, pseudomonads are more easily detected on mushroom species growing of compost (ex. *Agaricus bisporus*) rather than on xylophagous species

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(ex. *Lentinula edodes*). Francesca et al. (2018) indicated that *Pseudomonas* isolated from escarole and red chicory can be antibiotic resistant (especially to amoxicillin and ampicillin). Sorrentino et al. (2018) suggested a treatment with gallic acid and refrigeration to reduce pseudomonads loads and inhibit the growth of other undesirable microorganisms to elongate the shelf-life of truffles.

The natural truffle-grounds of Sicily do not limit the access to domestic or wild animals, because they are essential for the spread of truffle spores; thus, a fecal contamination of the collected *T. aestivum* fruiting bodies has to be considered. This risk is even amplified by the higher presence of insects during the harvest period, promoting the proliferation of enteric bacteria also because the black summer truffle develops in the humic layer of soil, where microbial activity is particularly high (Rivera et al., 2010). For this reason, enterococci, total coliforms, and members of Enterobacteriaceae family have been object of the present investigation. The total coliform levels registered in the present work are almost closed to those reported by Comi et al. (2010) for truffles collected in Molise region. The levels of Enterobacteriaceae showed in our work are higher than those found on truffles by other authors (Nazzaro et al., 2007; Reale et al., 2009; Rivera et al., 2011). Similar Enterobacteriaceae levels were also reported for wild and cultivated mushrooms (Venturini et al., 2011). This group of microorganisms determines a decay of the organoleptic and visual qualities of vegetables and includes species potentially pathogenic for humans (Alfonzo et al., 2018).

Our data showed cell densities of enterococci ranging between 3.00 and 5.15 log CFU/g, higher than the values showed by Comi et al. (2010). Clostridia, when present, were in the same order of magnitude of those reported by Comi et al. (2010) and Reale et al. (2009). Their presence may be associated with diseases, also serious, because they are able to synthesize toxins with different actions on the human health (Uzal et al., 2018). *Listeria* and CPS were not detected in any sample, while *E. coli* and *Shigella* spp. were found in a few samples. *Salmonella* spp. was not found in Sicilian samples, and only detected in truffles collected in Umbria region. All these strains represent a risk for the fresh consumption of truffles.

Regarding truffle associated eukaryotic microorganism, yeasts were found at variable levels in the different samples, but always higher than those found by Comi et al. (2010) and Rivera et al. (2011). On the contrary, mold levels, although variable among samples, were slightly lower than those reported by the previous works. Yeasts and molds during the storage of truffles might generate off-flavours and affect their visual quality, taste, and general acceptability (Rivera et al., 2010, 2011). To this purpose, LAB strains characterised by antifungal properties can be used to prevent the growth of molds on fresh truffles during the refrigerated storage (Sorrentino et al., 2013).

Conclusion

Although the microbiological characteristics of black summer truffles collected in woods and forests of various areas of Italy are known, this is the first survey aimed to characterize these products in Sicily. Specifically, this work evaluated the presence and cell densities of several microbial populations present on *T. aestivum* ascomata. Due to the mode of collection (e.g. contact with trained dogs) and post-harvest conditions applied during transport and storage, the contamination of black summer truffles does not exclusively depends on environmental factors.

The results of this work showed that total mesophilic populations were present at dominated levels and, in addition to alterative microorganisms (*Pseudomonas* spp. and LAB); potentially pathogenic microorganisms such as coliforms and members of Enterobacteriaceae (presumptive *E. coli*, *Shigella* spp., and *Salmonella* spp.) were detected. Therefore, a deepen identification of the dominant bacteria is necessary to determine their allotting into pathogenic species, in order to confirm that black summer truffles might represent a risk for human health, also considering the low amount used for food garnishing. However, based on the high levels of viable microorganisms detected, a decontamination treatment in addition to the common brushing is strongly suggested before truffle consumption.

Author contributions

N.F., L.S., and G.V. designed the project of study; F.C. conducted the experimental work; M.L.G. and D.D. did the statistical analyses; F.C. and L.S. wrote the manuscript. All authors read and approved the revised manuscript.

Conflicts of interest

All the authors declared that there was no conflict of interest in this study.

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