# Endogenous Bacteria of *Tuber aestivum* Ascocarps are Potential Biocontrol Agents of Microbial Post-harvest Deterioration of Truffles

**Neila Saidi, Shweta Deshaware, Ilef Ben Romdhane, Matab Nadim, Heikki Ojamo, Robert Kremer, Salem Shamekh**

***Abstract*— Most previous investigations of microbial interactions with truffle have been conducted within the soil environment and have not considered effects on post-harvested truffles. After harvest, truffles spoil easily and quickly within 4 days. This study evaluated the efficacy of bacteria isolated from fresh Tuber aestivum fruits ascocarps as biocontrol agents against the bacteria and fungi responsible for spoiling truffle fruit. Effects of acetic acid (12 %-v/v) and citric acid (10%-w/v) as inhibitors of isolated spoilage bacteria were also tested. An antagonism test between microbes extracted from unspoiled truffle microorganisms was performed against microbes responsible for spoilage of truffle fruits. Spoilage bacteria were identified.**

**Truffle fruits immersed in a supernatant from antagonistic bacteria culture medium contributed to non-spoilage and resulting in storage of fruit at room temperature for more than two weeks without spoilage development. In addition, acetic acid (12 %-v/v) inhibited all tested spoilage bacteria. However citric acid (10%-w/v) had no inhibitory effect on spoilage bacteria.**

**The results showed a high rate of antifungal activity among the bacterial isolates, indicating that truffle may be a common source for selection of microorganisms with important biotechnological potential, and may be useful for further biocontrol of food, plant, and soil-borne pathogenic bacteria and fungi.**

***Index Terms*— Aspergillus Actinomyces,, Bacillus, Micrococcus, Spoiling truffle, Serratia, Sphingomonas.**

## I. INTRODUCTION

Edible mycorrhizal mushrooms include some of the world's most expensive foods and have a global market of US$ billions [1]. In particular, truffles, the fruiting bodies of ascomycetes fungi belonging to the genus Tuber, are found worldwide and some of them have important commercial value due to their characteristic aroma [2]. Truffles are hypogeous fungi that form a symbiotic association within host plant roots in order to accomplish their complex life cycle [3]. 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 fruiting body (also called ascoma/ta, sporocarp/s or ascocarp/s) with asci and ascospores [4]. This cycle reportedly is influenced by bacteria present in both the mycorrhizosphere [5] and fruiting bodies of various truffle species [6]. Several microbiological approaches to characterise the bacterial populations of truffle have been performed, which clearly indicate that the bacterial community includes Pseudomonads, aerobic spore-forming bacteria, actinomycetes and rhizobiaceae [7, 8, 9, 10]. Truffle species possess common ecological features, such as a wide range of host plant species and a requirement for calcareous soil, but differ in their geographic distribution. *Tuber borchii Vittad.* and *Tuber aestivum Vittad.* are found throughout Europe [11]. *Tuber melanosporum Vittad.* is found in Italy, France and Spain, and *Tuber magnatum* Pico fruiting bodies, the Piedmont white truffle, found in northern Italy and the Istria Peninsula of Croatia.

Tuber species are ectomycorrhizal ascomycetes fungi which grow in association with different host trees such as oak, hazel and form hypogenous fruiting bodies known as truffles after a few years. Truffles are a hypogenous fungi usually harvested by trained truffle dogs. Some species of truffles, such as *Tuber magnatum*, *Tuber melanosporum*, and *Tuber aestivum*, are the most expensive edible fungi due to their organoleptic properties, especially the taste and unique aroma [12, 13, 14]. Traditionally produced in Italy, France, and Spain, truffles are considered to be a gourmet product and fresh truffles have the highest gastronomic value. The demand for fresh truffles has increased in the last decade but possibilities for a wider market are still limited by their short shelf life. Currently, postharvest technologies frequently used in other fresh products such as fruits, vegetables, and mushrooms are not applied to fresh truffles. The high market price of truffles worldwide (approximately 200 to 2000 €/kg) is one of the incentives for the development of food storage methods in order to extend fresh truffle shelf life and increase the possibilities for exporting to foreign markets. Dehydration, the process of senescence, and growth of superficial mycelium are the main factors that contribute to the rapid loss of organoleptic properties (spoilage) such as the texture, aroma, and taste of fresh truffles. In this context, the use of modified atmosphere packaging (MAP) could be considered as a possibility for postharvest storage of fresh truffles [15, 16] Rivera et al (2010) [17] have described the benefits of using MAP with microperforated films in the postharvest storage of fresh truffles (*Tuber melanosporum* and *Tuber aestivum*). The shelf life of *T. aestivum* is prolonged to 21 d and of *T. melanosporum* to beyond 28 d thereby increasing the possibilities for foreign export markets.

This study evaluated the efficacy of bacteria isolated from fresh *Tuber aestivum* fruits as biocontrol agents against the

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bacteria and fungi responsible for spoilage of truffle fruit and to test the effect of acetic acid (12 %-v/v) and citric acid (10%-w/v) on isolated spoilage bacteria.

## II. MATERIALS AND METHODS

**Ascocarps of *Tuber aestivum (T. aestivum)***

The ascocarps of *T. aestivum* was collected from Juva Truffle Center´s truffle orchard (Juva, Finland) with the help of a hunter dog (Fig 1). *T. aestivum* were dug out from natural truffle-grounds in October, 2014. The fresh collected truffles were individually wrapped to prevent cross-contamination, transported from the truffle-grounds to the laboratory in insulated boxes with ice packs and processed within a few hours.

The samples were brushed with a wet soft brush, rinsed with tap water, and forced air dried in a laminar cabinet. Qualitative selection of the carpophores was made by discarding truffles with soft texture, those parasitized as well as those extensively damaged during the harvest (by shovel or dog's teeth). The truffles were subsequently kept at 4 ◦C for 1 d until further investigations.

**Figure 1:** Photos of truffle ectomycorrhiza sampled from oak root systems from the square of Juva Truffle Center JTC with the help of trained *dogs* which are usually used for hunting the *truffles.*

The pH and electrical conductivity of the truffle suspension was determined by using a potentiometric electrode (720, Germany). The percentage of truffle dry matter content was determined after drying a fresh truffle ascocarp at 40°C until constant weight.

**Samples and the origin of spoiling microbial strains**

In this study, the spoiled surface white layer (extracted from truffle stored at room temperature for two days) and the spoiled green layer (extracted from truffle stored at room temperature for 5 days) contained the microorganisms responsible for spoilage of fresh truffles. Extraction of the microorganisms from spoiled truffle ascocarps was performed as described by Chand-Goyal and Spotts (1996) [18]. The isolated microorganisms from both layers of spoiled truffle were grouped into group 1 and group 2 respectively (Figure 2).

The microorganisms recovered from spoiled truffle fruit surfaces were suspended in 90 mL of sterilized physiological salt solution (0.9 % w/v NaCl) in 250 mL Erlenmeyer flask by agitation on a rotary shaker for 30 min at 240 rpm.

A 100 µL aliquot of each microbial suspension was spread-plated on tryptic soy agar (TSA) (Sigma, Germany) and yeast extract agar medium (YEAM). After the incubation for 1-2 days at 25°C, bacterial colonies which appeared on the plates with different morphotypes were individually isolated and cultured as single colonies on TSA and YEAM media. After incubation at room temperature, each isolate was inoculated on truffle ascocarps to verify its involvement in spoilage. Subsequently, the inoculated truffle ascocarp was pulverized to yield a suspension of $10^5$ spores/ml and $10^7$ CFU/ml for fungi and bacteria, respectively. After 24-48h of incubation at room temperature (approximately 25°C), microorganisms showing spoilage (expressed by layer spoiling developed on fruit surfaces) and release of specific volatiles served as the basis for identifying these isolates as spoilage microorganisms.

**Isolation procedure for antagonistic bacteria**

**Antagonism test in Petri dishes**

Group 3 bacteria consisted of bacteria extracted from the first layer of fruit from non-spoiled truffles. An antagonism test was performed with the bacteria belonging to Group 3 against Groups 1 and 2, respectively. An aliquot of 100 l of culture growth in TSB sampled from the spoilage bacteria or fungi was spread on the surface of PDA and each plate was then inoculated with five spots of antagonistic G3 bacteria.

After incubation at room temperature for 24-48h a clear zone (>0.5 cm) around the antagonistic bacteria indicated production of active substances involved in growth inhibition of the spoilage strains. A confirmative test was performed on the agar surface with a layer of inoculated fungi with the antagonistic bacterial strains individually applied in intersecting lines on the plates.

Isolates that markedly inhibited the growth of the spoilage fungi or bacteria were identified according to diagnostic guidelines listed in Bergey's manual (1984) [19].

**Kinetics of the inhibition of fungal spoilage**

Spores of fungi responsible for spoilage were enumerated periodically (5, 10, 30 min) after blotting the surface of PDA plates containing a 24-h culture of antagonistic bacteria spotted in the center of the plate.

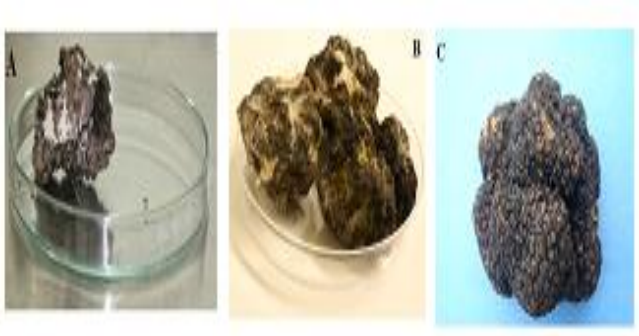


**Figure 2.** Photo of spoiled ***Tuber aestivum*** fruiting bodies stored at room temperature during only two days **A**, or 5 days **B**, providing the bacteria and fungi responsible for spoilage Group 1 and Group 2. Photo C presented Fresh and healthy ***Tuber aestivum*** fruiting bodies (truffle kept in freezer for at least 2h). The freeze fruiting body is submitted to a storage at

4°C for 6h followed by a second storage at room temperature approximately 22°C for 6 h before microorganisms extraction) providing bacteria and fungi named Group 3 probably antagonistic to spoiling microorganisms.

Samples of 100 µl of the fungal spore suspension flooding the Petri dish was taken at increasing distances (2, 4, 8 cm) from the spot of bacteria to the border of the plate. The 100 µl sample was diluted with 900 µl of physiological saline. Fungal spores were enumerated using a haemocytometer.

**Antagonistic test performed in truffle**

Selected isolates were transferred into 10 mL of TSB liquid medium in 100-mL Erlenmeyer flasks and incubated by shaking each culture at 100 rpm for 2 days under ambient conditions. Inhibitor activity against spoilage fungi by the bacterial suspensions was assayed at room temperature with truffle fruit pieces of 1x1 cm each placed in empty plastic Petri dishes. Each truffle fruit was drenched in a bacterial suspension (approximately $10^8$ CFU per fruit). After air drying, the fruits (treated and no treated) were spray-inoculated with a spore suspension of spoilage fungi (approximately $10^5$ spores/mL) and incubated at room temperature. Individual cultures and a consortium of the mixture of the three most efficient antagonistic bacteria were evaluated.

**Bacterial counts and identification**

Bacterial counts were determined by serial dilutions and plated onto tryptic soy agar after 48 or 72 h of incubation at 25°C. Bacteria colonies were roughly identified on the basis of their morphology and gram reaction and identification was considered based on diagnostic guidelines listed in Bergey's manual (1984) [19].

**Fungi identification**

The pure isolate of the fungi cultured on PDA medium was examined critically using prepared microscopic slides. Identification of the fungal species was performed with the aid of binocular compound microscope (10 and 40X) adopting the techniques used by Brugge (1977) [20]. Selective medium for Rhizopus recommended by Kordowska-Wiater et al. (2012) [21] was used: 0.2% (w/v) $KH_2PO_4$, 0.14% (w/v) $(NH_4)_2SO_4$, 0.05% (w/v) $MgSO_4 \times 7H_2O$, 0.03% (w/v) $CaCl_2$, 0.1% (w/v) yeast extract, 0.05% (v/v) microelements solution ($FeSO_4 \times 7H_2O$; $MnSO_4 \times H_2O$; $ZnSO_4$), 0.1% (v/v) Tween 80, 1.0% (w/v) sugar (sucrose, maltose, L-arabinose), 2.0% (v/v) agar, pH 5.5-5.7. Also, for Aspergillus strains the identification was to genus level by means of their morphological characteristics and replicated in Malt Extract Agar (Sigma-Aldrich) with the following composition distilled water 1000mL: malt extract 30.0 mycological peptone 5.0 Agar 15.0.

**Chemical treatment**

An initial chemical treatment for control of spoilage microorganisms comprised of 10 mL of 12 % acetic acid added to a fungal spore suspension of $10^5$/mL. Magnetic agitation was applied for 1min and 100 µL of the mixture was spread on the surface of PDA medium followed by incubation at room temperature for 5 days. Bacterial chemical treatment was applied to a pellet of 20 mL bacterial culture grown in TSB medium. The supernatant was discarded and an equal volume of sterilized water was added to each pellet. Ten milliliters of microbial suspension of approximately $10^7$ CFU/mL was adjusted to a final concentration of 10% v/v of acetic acid. After magnetic agitation for 1min, 100 µL of the mixture were spread on the surface of TSA medium followed by incubation at room temperature for 3 days. As a second treatment, the same protocol was applied by using citric acid 10% w/v instead of 12 % acetic acid.

Physio-chemical truffle characterization, antagonistic tests, the kinetics of fungi inhibition and chemical treatment were performed by using at least three repetitions.

## III. RESULTS

**Truffle fruit characteristics**

Results showed that the truffle fruit possess the following characteristics: pH=4.9 in 34 % suspension in water; dry matter content 24%.

**Spoilage bacteria**

The first group of spoilage microorganisms (G1) denoted with a suffix (BFS) extracted from a spoiled truffle fruit incubated for only 24h at room temperature included four different bacteria designated as BFS1, BFS2, BFS3 and BFS4. Also, mycelia extracted from truffles were isolated on PDA medium and denoted M. The second group of microorganisms (G2) obtained by extending the time for spoiling to 5 days included two fungi, F1 and F2 and other four bacteria B1, B2, B3 and B4 (Table 1).

**The spoiling effect of individual strains on truffle fragment**

Each bacterial suspension was pulverized at the surface of truffle fruit and incubated at room temperature. Results showed that all predicted spoilage microorganisms belonging to the first or the second group showed a spoiling effect when added individually to a truffle fruit body (Figure 3).

**First contact between F1 (spoiling fungi) and A1 (antagonistic bacteria)**

The first contact between the bacteria A1 and the spoiling fungus F1 by flooding surface PDA with a spore suspension showed abundant spores around the antagonistic bacterium after 5 min of contact. When the time is extended to 10 min of contact, the number of spores around the antagonistic bacterium was reduced and after half an hour the number of spores was further reduced around the antagonistic bacteria with all spores of spoiling fungi blocked towards the border of Petri dishes (Figure 4), showing production of active substances by the spoiling fungi.

Quantification of spores after contact with antagonistic bacteria showed clearly that the contact during only 2 min between the fungi F1 and the strain A1 was insufficient to reduce the number of spores in all samples considered at different distances from the center of the Petri dishes. However, extending the time of contact to 10 min or more reduced considerably the spores numbers mainly adjacent to antagonistic bacteria. The number of spores became more important in the border of Petri dishes (Figure 5).

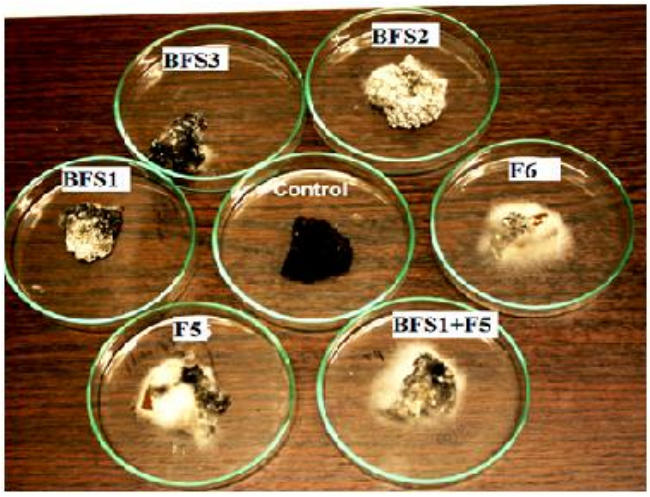


**Figure 3**. Photo of inoculated truffle fruits with each spoiling bacteria or fungi.

The control sample was not inoculated. The photo is taken 24h after inoculation and storage at room temperature. the photo presented only one repetition but the experiment was performed in triplicate.
BFS1, BFS2 and BFS3 indicated the first spoling bacteria. F5 and F6 Two fungi responsible for the later spoiling phenomena. BFS1+F5: indicated that the body truffle is inoculated by mixture of first spoling bacteria and fungi strain F5.

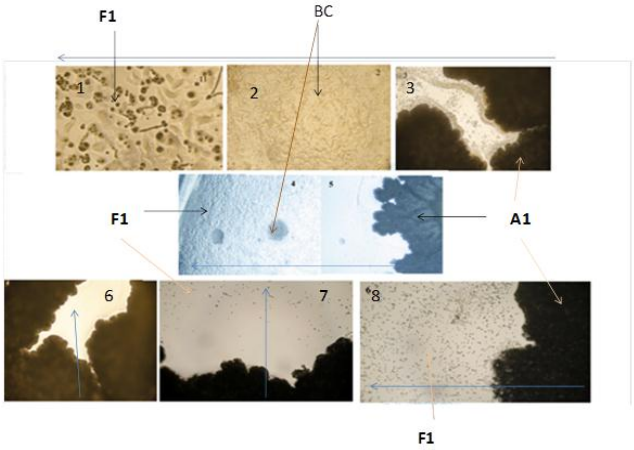


**Figure 4. Effect of first contact between F1 (spoiling fungi) and A1 (antagonistic bacteria)**
**1**: Spores of fungi F1 in association with active compound liberated by antagonistic bacteria A1; **2**: Active compound of antagonistic bacteria A1; **3**: Contact of A1 and F1 during 5 min; **4**: Active compound produced by F1 after 30 min of contact with A1; **6:** Contact of A1 and F1 during 30 min of contact with A1; **7**: Contact of A1 and F1 during 10 min of contact with A1. Bleu narrow indicated the direction in Petri dishes from the center to the border. The experiment was performed in triplicate and we choose only some photos to present. but after each inoculation of antagonistic bacteria the same phenomena was observed.

The first contact between the bacteria A1 and the spoiling fungus F1 by flooding surface PDA Petri dishes with a spore suspension showed abundant spores around the antagonistic bacterium after 5 min of contact. When the time is extended to 10 min of contact, the number of spore around the antagonistic bacterium was reduced and after half an hour there was reduction of the number of spores around the antagonistic bacteria and all spores of spoiling fungi were blocked towards the border of Petri dishes (Figure 5) and showed a production of active substance by the spoiling fungi.

The results indicate the production of two active compounds: the first one produced by the antagonistic bacteria situated at the center of the plates and the second one produced by the spoilage fungi close to the plate borders.

**Collection of bacteria isolated from truffles and their biotechnological properties**

A total of twenty-eight strains were isolated by culture-dependent methods from *T. aestivum* extracts.

All strains were tested for antagonism directed against spoilage bacteria. The most effective bacteria determined by the antagonistic test are presented in Table 2. Bacteria efficient in reducing growth of spoilage bacteria or fungi were identified. In addition, bacteria and fungi responsible for spoiling truffle were also identified.

Identification of bacteria, based on the Flow diagnostic guidelines [19] (Bergey, 1984), are presented in Table 1. Most of the first spoilage

**Table 1 .** Identification of spoilage bacteria and fungi, antagonistic bacteria and effect of 12% v/v acetic acid and 10% w/v citric acid.

| | Strain | Species | Acetic acid (12 %-w/v) | Citric acid (10%-w/v) |
|---|---|---|---|---|
| Spoiling Bacteria/Fungi | BFS1 | *Micrococcus luteus* | + | - |
| | BFS2 | *Staphilococcus aureus.* | + | - |
| | BFS3 | *Micrococcus varians* | + | - |
| | BFS4 | *Enterococcus faecalis* | + | - |
| | M | Mycelia of the *T. estivium* | + | - |
| | F1 | *Rhizopus sp* | + | - |
| | F2 | *Aspergillus sp* | + | - |
| | B1 | *Micrococcus varians* | + | - |
| | B2 | *Serratia fonticola* | + | - |
| | B3 | *Micrococcus luteus* | + | - |
| | B4 | *Streptococcus spp.* | + | - |
| | | *Group B (Streptococcus agalactiae)* . | | |
| Antagonistic Bacteria | 10 | *Bacillus sphaericus* | + | - |
| | 169 | *Actinomyces sp* | + | - |
| | 191 | *Sphingomonas sp* | + | - |

Identification of bacteria based on flow Charts in Bergey's Manual of Determinative Bacteriology 1984 [19], BFS: Bacteria first step spoiling means bacteria responsible for early spoilage which appeared within two days incubation at room temperature BFS1, BFS2, BFS3, BFS4. B1, B2, B3, B4 bacteria later step spoiling. F6, F5 fungi later spoiling,

bacteria belong to gram positive cocci strains except the strain B2 which is gram negative.

**Collection of microorganisms isolated from truffles and their biotechnological properties**

The first spolage bacteria BFS1, BFS2, BFS3 and BFS4 were identified as *Micrococcus luteus Staphylococcus aureus, Micrococcus varians* and *Enterococcus faecalis,* respectively. However B1, B2, B3 and B4 spoilage bacteria, which appeared later (after 5 days truffle incubation at room temperature) were identified as *Micrococcus varians, Serratia fonticola, Micrococcus luteus and Streptococcus agalactiae,* respectively. Two fungi responsible for later spoilage were *Rhizopus sp.* and *Aspergillus sp.* (Figure 6). Mycelia of *T. aestivium* (M) was also isolated.

**Table 2.** Antagonism test by bacteria provided from no spoiled fruit (antagonistic strain) and bacteria responsible for spoiling truffle fruit.

| Antagonistic strains | Spoilage strains | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | BFS1 | BFS2 | BFS3 | BFS4 | M | F1 | F2 | B1 | B2 | B3 | B4 |
| A1 | ++ | - | - | - | - | +++ | +++ | - | +++ | - | + |
| A2 | - | - | - | ++ | - | - | - | - | - | +++ | - |
| A3 | - | ++ | - | ++ | - | - | - | - | - | - | - |

Indicator strains means spoiling bacteria. BFS: Bacteria first step spoiling means bacteria responsible for early spoiling which appeared within two days incubation at room temperature BFS1, BFS2, BFS3, BFS4. B1, B2, B3, B4 bacteria later step spoiling. F6, F5 fungi later soiling, Producers bacteria means antagonistic bacteria able to limit the growth of spoiling fungi or bacteria. -: no Absence of inhibition zone, +: inhibition zone is under 2mm, +++: inhibition zone is over 0.5mm. *Results indicate mean of three independent experiments.

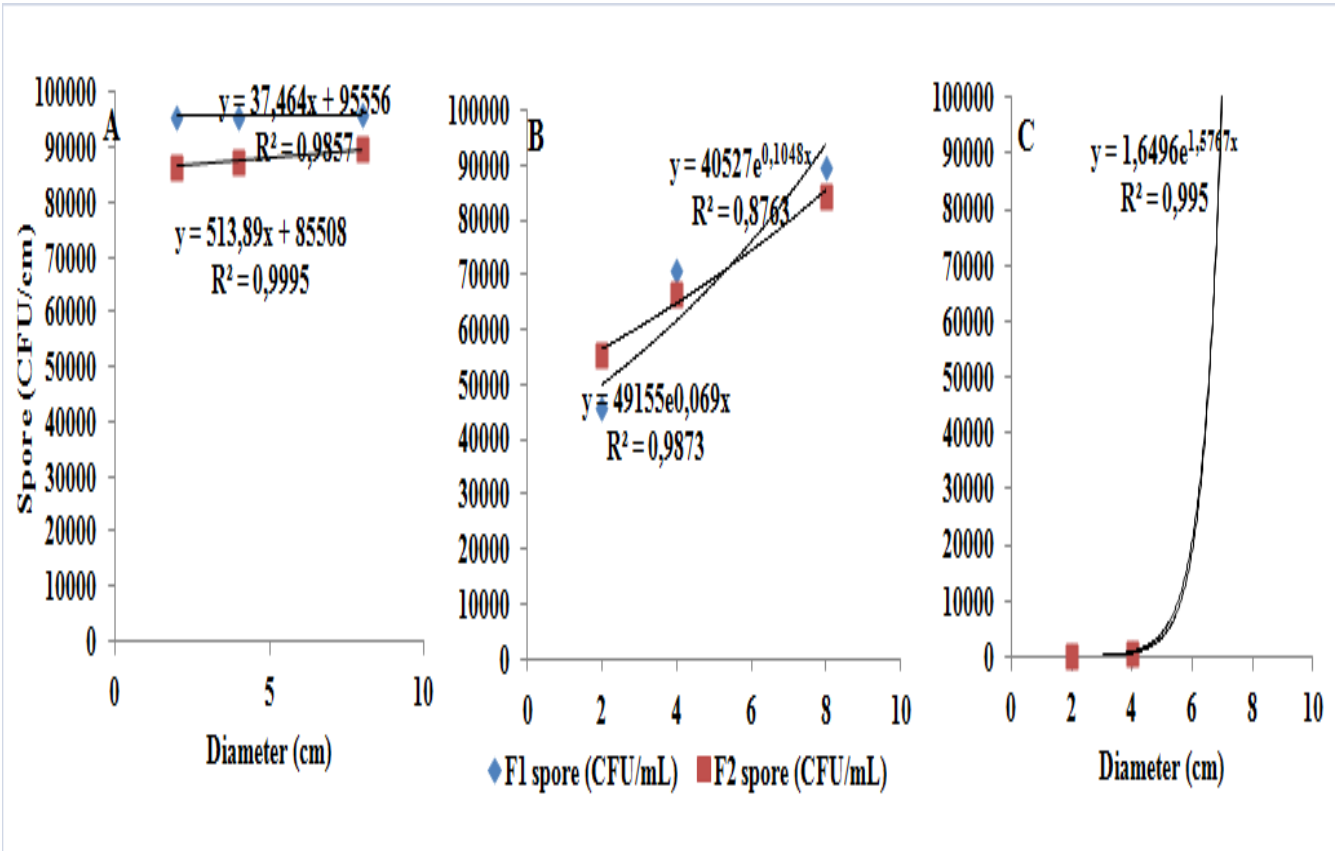


**Figure 5.** Enumeration of fungal spores responsible for spoiling effected during increasing time (5 min : **A**, 10 min **B**, 30 min: **C**) after blooding the Petri dishes containing a previous 24h culture of antagonistic bacteria A1 put in spot at center of the Petri dish.

The sampling of 100 µl from the sporal fungal suspension was considered at increasing diameter (2, 4, 8 cm) from the spot of bacteria. The sampled 100 µl was diluted in 900 µl of physiological water. Then, spores enumeration was considered by counting with a heamacytometer.

For the gram negative bacterium B2 responsible for spoilage of truffle, biochemical analysis showed the following characteristics: oxidase negative, lactose fermentation positive, MR-VP positive and $H_2S$ negative. Based on these characteristics, strain B2 was identified as *Serratia fonticola* using Bergey's manual (1984) [19].

The three bacteria antagonistic against most spoilage bacteria were identified as: strain A1, *Bacillus subtilis*; strain A2, *Actinomyces* sp; and strain A3; *Sphingomonas* sp. The primary spoilage bacteria may serve to develop a favorable micro-environment for establishment of succeeding spoilage bacteria or fungi.

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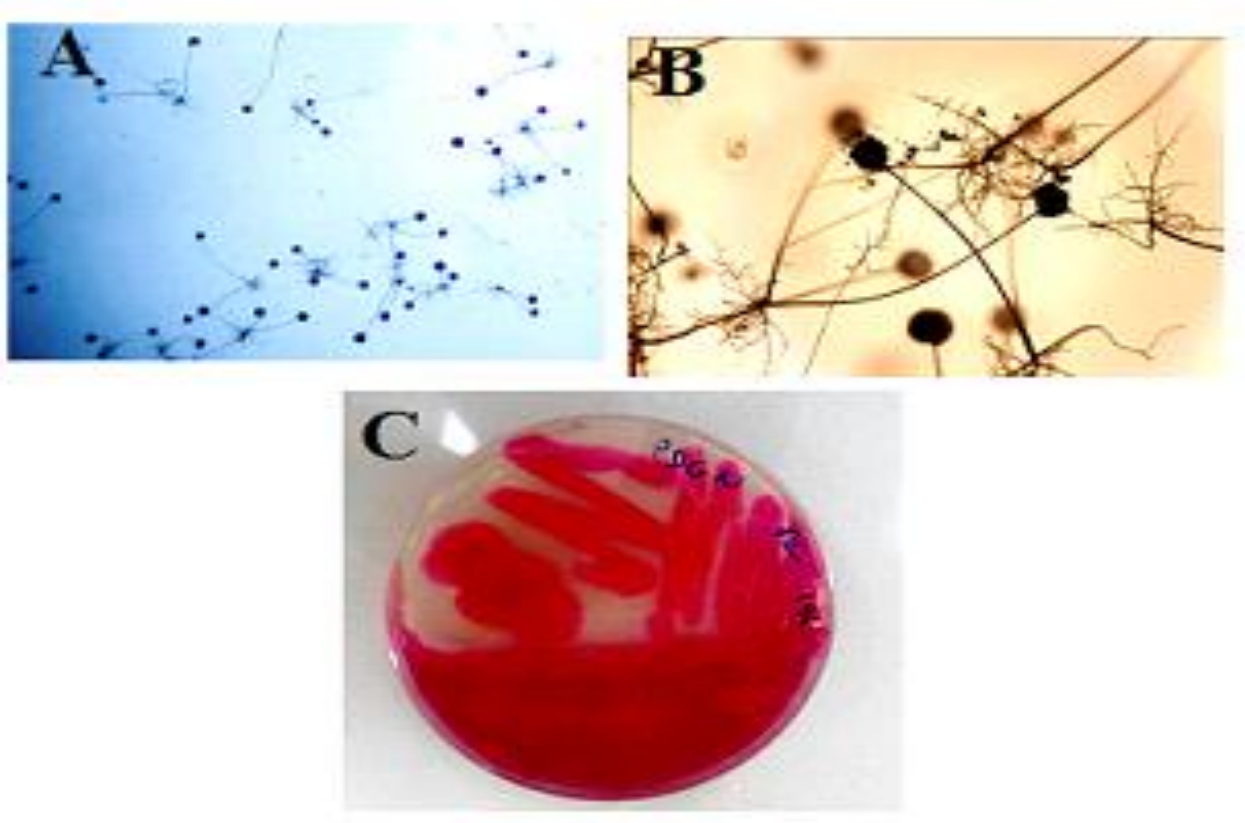


**Figure 6**. **Aspect of phenotypes of spoiling fungi F1 and Serratia strain isolated from *T. aestivum* ascocarps.** **A:** range of conidiophores phenotypes, which phylogenetically belong in Aspergillus (One example of Fungi responsible for *T. aestivium* spoiling) experiment performed at room temperature approximately 25 °C. Photo examined microscopically with x10 and x40 objectives, respectively. **B:** *Serratia fonticola* isolated from *T. aestivum* ascocarps **C**: Serratia strain isolated from *T. aestivum* ascocarps.

**Antagonism test with truffle fruits**

When truffle fruit was dipped in individual supernatant or the consortium culture composed of three bacteria inhibitory toward at least one fruit spoilage pathogen, and incubated at room temperature for three days, spoilage development was eliminated and moisture content was reduced (Figure 7).

**Effect of acids on spoiling fungi and bacteria isolated from *T. eastivium***

Acetic acid (12 %-v/v) possesses a high inhibitory effect on all isolates of spoilage bacteria. However, citric acid (10%-w/v) applied to spoilage bacteria showed no inhibitory effect (Table 1)**.** Following these treatments confocal microscopy revealed that citric acid changed the shape of fungal hyphae and their nuclear distribution. In fact, spore mixtures supplemented with acetic acid exhibited increased lengths of the hyphal tips, while more intense branching was observed in untreated mycelia (Figure 7).

**DISCUSSION**

Originally, fresh truffle fruits possess a relatively low pH of 4.9 and the natural occurrence of organic compounds provides suitable conditions for the growth of acidophilic microorganisms. Common bacteria that cause spoilage are acetic acid bacteria, lactic acid bacteria and spore forming bacteria. Also most fungi thrive under acidic pH conditions. Kruzselyi and Vetter (2014) [22] analyzed the dry matter of *T. aestivum* fruits and reported that the fruit contents contained 19.11% crude protein; 2.27% crude fat; 22.03% crude fibre; 7.55% crude ash, 10.63% chitin, 48.9% carbohydrates: and 92.44% total organic constituents expressed on dry weight basis. The richness of *T. eastivium* in carbohydrates and crude protein may be the basis for microbial spoilage under the acidic micro-environment provided by the truffle fruit.

In fact, the truffle tuber is harvested from soil in September when temperature is favorable for many microorganisms thus the presence of bacteria in truffle is expected.

Among the truffle spoilage fungi, *Rhizopus spp.* are known to detrimentally affect other economically important food plants reported by its profusion in both tomato plants and potato in the field and during storage of the harvested products [23].

The presence of mycotoxin-producing *Aspergillus* species in food is considered a worldwide public health problem [24]. *Aspergillus spp.* are predominant in many food spoilage cases including in coffee beans in which the fungi can potentially produce toxins such as ochratoxin [25, 26].

Species of *Bacillus* have long been troublesome to food producers on account of their resistant endospores. Regarding truffle, previous research noted that *Bacillus*, and other closely related species were present in the brûlé zone. In fact, the development of the *Tuber melanosporum* mycorrhizal symbiosis is associated with the production of an area devoid of vegetation (commonly referred to by the French word 'brûlé').

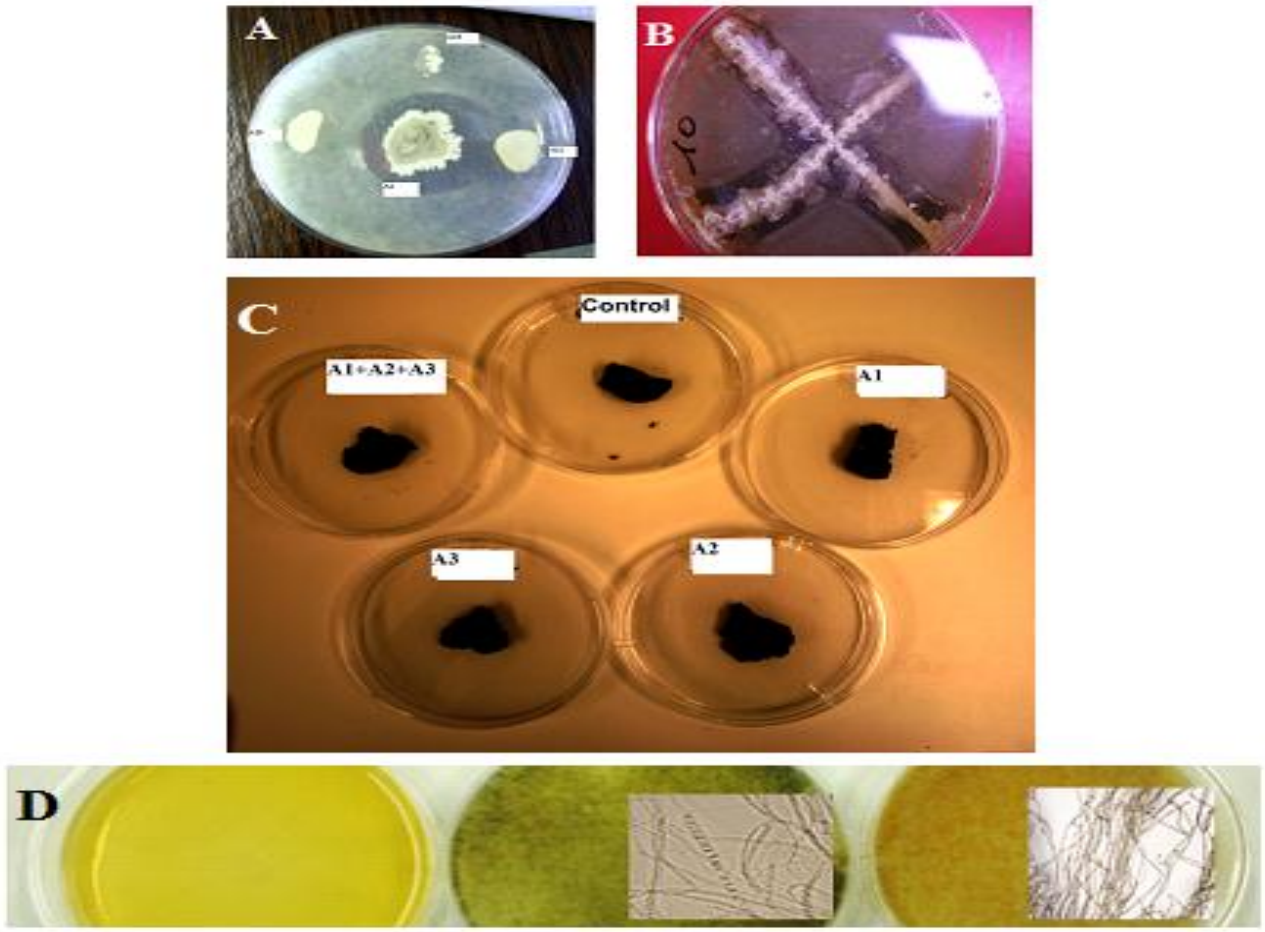


**Figure 7**: Photos illustrating the antagonism test between fungi F1 and different antagonistic bacteria accompanied by photos of chemical treatment.

**A:** Photo of antagonism between some of bacteria isolated from no-spoiled truffle denoted with suffix A and spoiling fungi strain F1. Test was performed in PDA plates and each inoculated plate received five spot from the antagonistic bacteria. After incubation at room temperature for 24-48h a clear zone around the antagonistic bacteria indicated production of active substances able to kill the spoiling strains. **B**: Photo of the second confirmative test of the antagonistic bacteria individually set not in spot but in cross lines in shape X. **C:** Photo of truffle fruits immersed in a mixture of supernatant of bacteria able to limit the growth of the spoiling fungi. Control present the sample immersed in a sterilised potato dextrose broth. The photo is taken after two weeks of storage at room temperature after the inoculation. **D:** Photo of fungi F1 treated with (from left to right) acetic acid **12% v/v**, Control (without any previous treatment) citric acid 10% w/v. Volume of 10 mL of acetic acid 12 % was added to fungal sporal suspension $10^5$/mL. Magnetic agitation was applied for 1mn and 100 µL of the mixture were spread at surface of PDA medium. Photo is performed after 5 days incubation at room temperature. Microscopic photo (X40).

In these brûlé zones fungal communities as well as other microorganisms are affected by *T. melanosporum*. Firmicutes (e.g., *Bacillus*), several genera of *Actinobacteria*, and a few *Cyanobacteria* are more dominant inside the brûlé compared with outside [14]. Truffle Brûlés have an impact on the diversity of soil bacterial communities.

The presence of the *Bacillus subtilis* strain may have originated in the soil around the truffle ascocarps. Generally, the subtilis group (*Bacillus licheniformis*, *Bacillus subtilis*, and *Bacillus pumilus*), is associated with a range of clinical conditions, food spoilage such as ropy bread, and incidents of food-borne gastroenteritis [31]. *B. licheniformis* is a common contaminant of dairy products and has also been associated with food poisoning in humans, as well as with bovine toxemia and abortions [32]. Other studies showed that some *Bacillus* species may limit selected pathogenic fungi [33].

The first results of the present study showed that *T. aestivum* may provide interesting microbial strains to be exploited in biotechnological applications. We also found that *T. aestivum* included spoilage bacteria able to negatively affect health and the environment. Contamination of fruits and vegetables generally is caused by bacteria including species of *Bacillus, Enterobacter, Lactobacillus, Leuconostoc, Pseudomonas, Sarcina, Staphylococcus,* and *Streptococcus*. Various molds and yeasts also inhabit the fruits and vegetables. The presence of one specific group of bacteria or others depend firstly on substrates present in the fruit body and on soil properties. Many members of the *Serratia* genus have been isolated from rhizospheres and shown to confer positive inputs such as Plant Growth Promoting Rhizobacteria (PGPR) traits, including solubilization of inorganic phosphate [34] and phytohormone production and phytoremediation [35], and protection of plants from flood-induced damage [36]. They also improve plant health indirectly by reducing plant pathogens of bacterial, fungal, and nematodal origin [37, 38]. These

benefits may be exploited in the future to improve the quality of truffle in its natural environment or in storage. Also, Rauha et al. (2000) [39] showed that some Finnish plant extracts may act against many spoilage bacteria such as *Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus aureus* and *Staphylococcus epidermidis*. It will be interesting to test the plant extracts on truffle fruit to extend storage time, since the major spoilage bacteria existing in Truffle fruit were affected by these extracts.

Most acidic fruits host the same groups of spoilage bacteria. For truffle spoilage, there are a few studies on the effects of various storage treatments for most of the common edible truffles. The change in the aroma is certainly influenced not only by soil characteristics, but depend also on bacteria and yeasts that live in the soil around truffle ascocarps. In fact, pure cultures of yeasts isolated from fruiting bodies of *T. melanosporum* and *T. magnatum* produce VOCs characteristic of each truffle.

Post-harvest decay control strategies often involve consideration of the following critical factors: type of spoilage organism involved, location of the pathogen, best time for application of the treatment, maturity of host, as well as external conditions such as environment during storage and transportation [40].

Weak organic acids such as acetic, lactic, benzoic, sorbic and citric acid have been traditionally used as preserving agents. These molecules inhibit growth of bacterial and fungal cells while sorbic acid has been reported to inhibit germination and outgrowth of bacterial spores [41]. Acetic acid at concentrations of 20–200 mM and 20–80 mM have been shown to induce programmed cell death in exponentially growing *S. cerevisiae* [42] and Z. bailii, respectively [43].

Acetic acid (pure) has been effectively used for treatment in vapour form [44] or as vinegar [45] for reducing post harvest decay. Fumigation with acetic acid has been carried out on apples, grapes, kiwifruit, pears, tomatoes [46], and stone fruit [47] to prevent decay caused by *B. cinerea, P. expansum or M. fructicola.*

Ryu *et al.* (1999) [48] studied the effect of reduced pH over a range with combinations with different organic acids on *E. coli* O157:H7 and found that acetic acid was the most lethal and effective acid, followed by lactic, citric and malic acid. However Hsiao and Siebert, (1999) [49] reported that lactic acid was more lethal than acetic acid for *E. coli* O157:H7.

Delaquis *et al*. (1999) [50], studied the effect of fumigation of acetic acid (242 µl of acetic acid per liter of air for 12 h at 45°C) on mung bean seeds inoculated with *S. typhimurium*, *E. coli* O157:H7, and *L. monocytogenes* (3 to 5 log CFU/g) and found that it significantly inhibited the growth of *S. typhimurium* and *E. coli* O157:H7.

Antimicrobial action of acetic acid has also been shown in salmonella species both *in vitro* and on cut surfaces of apple slices [51]. Dipping lettuce in 1% citric acid in combination with 3 ppm ozone for 1 minute resulted in 2.31 - and 1.84-log reductions ($P < 0.05$) for *E. coli* O157:H7 and *L. monocytogenes* respectively [52]. Our results appeared in contradiction with those obtained by Shokri (2011) [53] who showed that citric acid has a significant antifungal activity against all pathogenic fungi tested (*Trichophyton mentagrophytes, Candida albicans, Aspergillus funigatus and Malassezia furfur*. Bjornsdottir et al. (2006) [54] studied the protective effects of different organic acids on survival of *E. coli* O157:H7 at concentrations between 5 and 10mM and found that acetic, malic and l-lactic acid can have protective effects (1- to 2- log survival) whereas citric acid did not exhibit any protective effect at similar concentrations. Citric acid has been used in synergism with different spices such as pepper by decreasing the pH and hence potentiating anti-bacterial effects [55].

Food spoilage is a complex process and excessive amounts of foods are lost due to microbial spoilage despite modern day preservation techniques. Despite the heterogeneity in raw materials and processing conditions, the microflora that develops during storage and in spoiling foods can be predicted based on knowledge of the origin of the food, the substrate base and a few central preservation parameters such as temperature, atmosphere, water content and activity, and pH. Based on such knowledge, more detailed sensory, chemical and microbiological analyses can be carried out on the individual products to determine the actual specific spoilage organism. Whilst the chemical and physical parameters are the main determining factors for selection of spoilage microorganisms, a level of refinement may be found in some products in which the interactive behavior of microorganisms may contribute to their growth and/or spoilage activity.

For truffle, the ascocarps are harvested from soil and, in their natural ecological medium, several bacteria were able to produce volatiles in a limited area around the truffle. This area is known as the brulé area. In soil, it was shown that truffle-associated bacteria may improve truffle nutrition via their ability to solubilize phosphate, iron, and other nutrients [28]. Previous studies on cultivable truffle ascocarps-associated bacteria have focused on *Pseudomonas* and *Bacillaceae* strains, and provided evidence that these species may be involved in ascocarp degradation and spore release, or mycelium differentiation [56, 8, 9] .

Barbieri *et al.* (2007) [7] published a comprehensive report on the molecular diversity of bacteria inhabiting the ascocarps of *T. magnatum*. Truffle ectomycorrhiza and fruiting bodies harbor a diverse microbial community including bacteria, yeasts and filamentous fungi [6, 7, 57].

Most microorganisms that are initially observed on whole fruit or vegetable surfaces are soil inhabitants, members of a very large and diverse community of microbes that collectively are responsible for maintaining a dynamic ecological balance within most agricultural systems [58].

In this study, we reported for the first time the isolation and characterization of bacteria antagonistic to truffle spoilage microorganisms. The results showed high antifungal activity among the isolates, indicating that truffle may be a common source for the selection of microorganisms with important technological potential, which may be useful for further biocontrol of food-, plant-, and soil-borne pathogenic bacteria and fungi. Further investigations to elucidate the nature of inhibiting compounds should serve as a source of bioactive compounds able to be exploited in the future to not only extend the storage time of truffle fruit but also to be applied in many other areas of biotechnology.

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**Conflict of interest**

All authors disclosed any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations

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