Food Microbiology 84 (2019) 103251

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Are bacteria responsible for aroma deterioration upon storage of the black truffle *Tuber aestivum:* A microbiome and volatilome study

Maryam Vahdatzadeh[a,b], Aurélie Deveau[c], Richard Splivallo[a,b,*]

[a] *Institute of Molecular Biosciences, Goethe University Frankfurt, 60438, Frankfurt am Main, Germany*
[b] *Integrative Fungal Research Cluster, 60325, Frankfurt, Germany*
[c] *Institut national de la recherche agronomique (INRA), Unité Mixte de Recherche 1136 INRA-Université de Lorraine, Interactions Arbres/Microorganismes, Centre INRA-Grand Est-Nancy, 54280, Champenoux, France*

ARTICLE INFO

Keywords:
Tuber spp.
Microbiome
Aroma
Volatile
Bacteria
Storage

ABSTRACT

Truffle fungi, luxurious food items with captivating aromas, are highly valued in the culinary world. However, truffles are perishable and their aroma undergoes deep changes upon storage. Additionally, truffle aroma might be partially derived from microbes. Hence, we investigated here the influence of storage on two factors, namely the volatile profile and bacterial community composition in the black truffle *Tuber aestivum.* The possible linkage among those factors was further explored.

Our results demonstrate important changes in the volatile profiles of truffles over nine days of storage at room temperature. In the same time frame, dominant bacterial classes characteristic of fresh truffles (*α-Proteobacteria*, *β-Proteobacteria*, and *Sphingobacteria* classes) were gradually replaced by food spoilage bacteria (γ-*Proteobacteria* and *Bacilli* classes). Freshness and spoilage volatile markers (i.e. dimethyl sulfide (DMS), butan-2-one, 2- and, 2- and 3-methylbutan-1-ol, and 2-phenylethan-1-ol) were identified. Lastly, network analysis showed correlations between those markers and specific bacterial classes typical of fresh and spoiled truffles.

Overall, our results demonstrate the profound effect of storage on the aroma and bacterial community composition of truffles and highlight how the gradual replacement of the commensal microbiome by spoilage microbes mirrors shifts in aroma profile and the possible loss of fresh truffle flavor.

1. Introduction

Truffles (*Tuber* spp.) are the fruiting bodies of *Ascomycete* fungi that develop underground in close association with the roots of trees and shrubs (Fassi and Fontana, 1967; Selosse et al., 2017). Unique organoleptic properties confer truffles the status of standalone luxury food often served in the most prestigious restaurants (i.e. the white truffles *Tuber magnatum,* or the black truffles *Tuber melanosporum* and *Tuber aestivum*) (Splivallo and Culleré, 2016). Worth thousands of euros per kilogram, truffle prices are exorbitantly high, partially owing to limited seasonal availability (a few months per year), a short shelf-life (1–2 weeks) and the lack of proper preservation methods that would keep aroma intact.

Truffle aroma is made of hundreds of volatile compounds (hydrocarbons with various functional groups and sulfur atoms) (Culleré et al., 2010; Liu et al., 2012; Splivallo and Ebeler, 2015) of which, as with other food, only a small percentage is detectable by humans (Dunkel et al., 2014). In the specific case of truffles, 15 to 20 aroma active compounds (odorants) per species are responsible for the typical truffle smell perceived by humans (Culleré et al., 2010; Schmidberger and Schieberle, 2017). Some of these odorants are common to several truffle species (i.e. 2- and 3-methylbutanal, 2- and 3-methylbutan-1-ol and oct-1-en-3-ol), whereas others are species-specific (i.e. 2,4-dithiapentane in *T. magnatum*, thiophene derivatives in *T. borchii*) (Fiecchi et al., 1967; Splivallo et al., 2011; Splivallo and Ebeler, 2015).

Truffle aroma is deeply affected by storage as highlighted in the scientific literature (Aprea et al., 2007; Bellesia et al., 1998; Culleré et al., 2013; Falasconi et al., 2005; Pennazza et al., 2013; Splivallo et al., 2015). For instance, the concentration of dimethyl sulfide (DMS), a key contributor to truffle smell in many truffle species (Splivallo et al., 2011), was shown to decrease in fruiting bodies of the black truffle *T. melanosporum* during two months of cold storage (Culleré et al., 2013). By contrast, the concentration of other sulfur-containing volatiles such as thiophene derivatives characteristic of *T. borchii* fruiting bodies were reported to increase within weeks at 25 °C but remained unchanged at 0 °C (Bellesia et al., 2001). Besides temperature, several other

---

[*] Corresponding author. Institute of Molecular Biosciences, Goethe University Frankfurt, Max-von-Laue-Str. 9, 60438, Frankfurt am Main, Germany.
E-mail address: richard.splivallo@a3.epfl.ch (R. Splivallo).

https://doi.org/10.1016/j.fm.2019.103251
Received 8 April 2019; Received in revised form 4 June 2019; Accepted 22 June 2019

Table 1
Detailed information about *T. aestivum* fruiting bodies.

Origin	Truffle orchard/Host trees	Harvest	Truffle 1	Truffle 2	Truffle 3
[Region, Country]	[Natural or Artificial/Species]	[Month Year]	[Weight/Maturation]		
Puy-de-Dôme, France	Natural/*Quercus* spp. & *Corylus* spp.	July 2016	48g/70%	17g/67%	19g/12%
Var, France	Natural/unknown	July 2016	51g/10%	36g/78%	33g/64%
Dordogne, France	Natural/*Corylus* spp.	July 2016	27g/88%	24g/55%	18g/60%
Wallis, Switzerland	Artificial/*Pinus nigra* & *Quercus robur*	November 2016	40g/100%	11g/81%	13g/95%

Maturity ranging from 0 (fully immature) to 100 percent (fully mature) (maturity was determined as described earlier (Splivallo et al., 2012)).

preservation techniques (i.e. sterilization, freezing, freeze-drying, hot-air drying, canning, gamma-ray irradiation, and modified atmosphere packaging) have been applied to truffles for shelf-life extension (Campo et al., 2017; Culleré et al., 2012, 2013; Nazzaro et al., 2007; Palacios et al., 2014; Reale et al., 2009; Rivera et al., 2010b, 2011a; 2011b; Saltarelli et al., 2008). For instance, Campo et al., (2017) compared the influence of canning, hot air-drying, freezing and freeze-drying on the aroma composition of the black truffle *T. melanosporum* and concluded that freeze-drying was the best method to preserve the "fresh" aroma of *T. melanosporum* (Campo et al., 2017). Yet, according to another study, freeze-drying caused substantial changes in the flavour of *T. aestivum* (Palacios et al., 2014). These contrasting results highlight that a single preservation technique might not be appropriate for all truffle species.

Truffle fruiting bodies harbour complex commensal microbial communities, overall dominated by bacteria that can reach densities of billions of bacterial cells per gram of truffles fruiting bodies (Barbieri et al., 2007, 2005; Gryndler et al., 2013; Rivera et al., 2010a; Sbrana et al., 2002; Vahdatzadeh et al., 2015). Comparing bacterial communities in the soil of truffle orchards or at the surface (peridium) or within (gleba) fruiting bodies of *T. melanosporum* revealed similarities between bulk soil and peridium but stark contrasts between peridium and gleba (Antony-Babu et al., 2014). Additionally, the characterization of the bacterial communities colonizing the gleba of many truffle species (i.e. *T. aestivum, T. melanosporum*, *T. borchii*, *T. magnatum*) evidenced the presence of a "core truffle microbiome" predominantly made of bacteria of the α-*Proteobacteria* class and the *Bradyrhizobium* genus (Antony-Babu et al., 2014; Barbieri et al., 2007, 2005; Benucci and Bonito, 2016; Splivallo et al., 2015; Vahdatzadeh et al., 2015). Recently, however, extensive sampling of a large number of *T. aestivum* fruiting bodies in two distant orchards highlighted that about 10–20% of truffles were predominantly colonized by members of the β-*Proteobacteria* or *Sphingobacteria* classes instead of α-*Proteobacteria* (Splivallo et al., 2019).

As with most artisanal and non-sterile food products, storage conditions most likely affect microbial communities living within truffles. This has only been addressed in a few cases using culture-dependent methods. For instance, the population of cultivable bacteria rapidly grew by two orders of magnitude in fresh fruiting bodies of *T. borchii*, *T. melanosporum* and *T. aestivum* stored at 4 °C after 15 days (Saltarelli et al., 2008). Similarly, a steady increase in the population of spoilage bacteria of the *Enterobacteriaceae* family was observed in *T. aestivum* conserved at 4 °C during 21 days (Rivera et al., 2010b). The latter studies documented shifts during storage in the fraction of cultivable bacteria colonizing truffles. Yet, how these observed changes affect the overall truffle microbiome or whether distinct starting microbiomes (i.e. α-*Proteobacteria*, β-*Proteobacteria* or *Sphingobacteria*) react differently to food spoilage bacteria remains unknown. Neither is it known how shifts in microbial populations impact truffle aromas.

The aim of this study was threefold. Our first aim was to assess the extent to which storage at room temperature affected microbiome and volatilome of truffle fruiting bodies of the black truffle *T. aestivum.* To do so, changes in the volatile profiles and bacterial community structures were measured by GC/MS and high-throughput amplicon sequencing. A second aim was to identify freshness and spoilage "markers" (bacteria or volatiles) that might be useful to assess truffles' quality. The third aim was to highlight microbial groups that might be particularly relevant in modifying truffle aroma during storage by correlating the concentration of single volatiles to the relative proportions of microbes.

2. Material and methods

2.1. Biological material

A total of twelve *Tuber aestivum* fruiting bodies were collected from four different geographical regions that included three truffle-orchards in France and one in Switzerland (three truffles per each location, see Table 1 for details). Each truffle was washed and the outer part (peridium) was peeled off to focus in this work on the microbiome of the gleba, which is the most characteristic of truffles as highlighted in the introduction (Antony-Babu et al., 2014; Vahdatzadeh et al., 2015). The inner part of the fruiting body (gleba) was divided into four subsamples of comparable size. Volatile fingerprinting and DNA extraction (for microbiome analysis) was performed either immediately (one subsample, t = 0 days of storage) or after storage of 3,6 or 9 days at room temperature. Specifically, for the storage trials, the three subsamples per truffle were placed in a 50 ml tube, the tube was closed with a screw cap and stored in the dark. Subsamples were then removed at days 3, 6 and 9 and processed as described hereafter. Even though truffles are generally stored at cold temperatures, room temperature was used here to speed up the spoilage process and maximize microbial shifts as well as the drift in the volatile profile. Under these conditions, samples clearly spoiled within 9 days and emitted an unpleasant smell at the last time point.

2.2. Volatile profiling of T. aestivum fruiting bodies during storage and identification of truffle freshness and spoilage markers

Volatile compounds of gleba samples (300 ± 5 mg) were analyzed using solid-phase microextraction gas chromatography-mass Spectrometry (SPME-GC/MS) as described earlier (Vahdatzadeh and Splivallo, 2018). All the volatile analyses were performed with three technical replicates per fruiting body (3 × 300 mg samples per truffle and time point). GC/MS output peaks were aligned using Tagfinder software version 4.1 (Luedemann et al., 2011) using the following parameters: Timescale: 2, Low Mass: 40, High Mass: 400. Peak finder tool; Smooth Width Apex Finder: 1, Low Intensity Threshold: 4500 (Smooth Apex), Smooth Width ± Apex Scan: 1 (Merge Peaks), Max Merging Time Width 1.0 (Large File Mode). Peak alignment; Time Scan Width 2.0; Gliding Median Group Count 1; Min Fragment Intensity 50. Tagfinder resulted in a matrix containing mass tags (the intensity of particular masses within certain retention time intervals) in rows for all samples in columns. Background noise was removed by removing any signal that was less than three times the signals detected from empty SPME vials. Additionally, any signal emitted from the 50 ml tubes, used for storage, was similarly removed from the aroma profile of the fruiting bodies. Subsequently, normalization was conducted by dividing the intensity of each tag to the total ion current (TIC) for each sample. The non-parametric Kruskal-Wallis statistical test ($p < 0.05, \alpha = 0.05$) performed in R (version 3.2.3) (Gentleman and Ihaka, 1996) was used

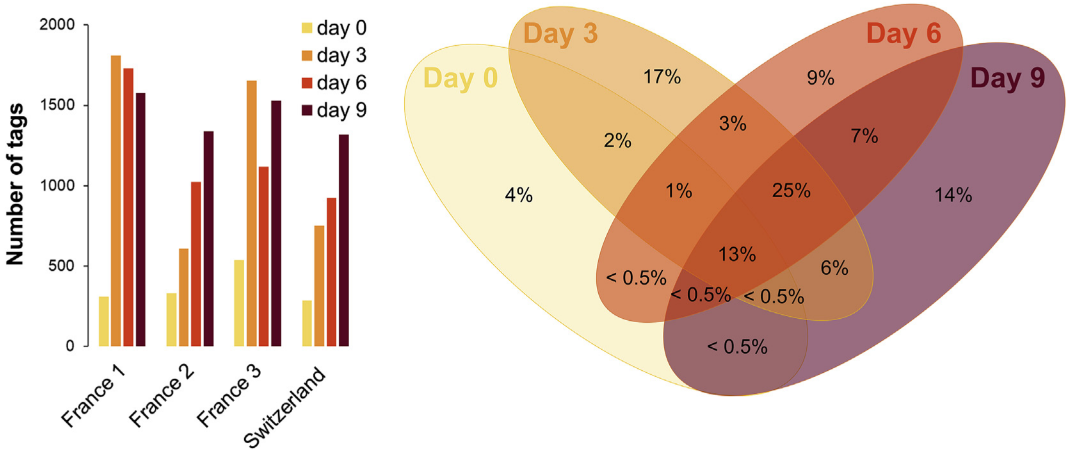

Fig. 1. Changes in the volatile profiles of *T. aestivum* fruiting bodies during storage. The bar chart illustrates the total number of volatile signals (TAGs) detected at each time point and site in at least one truffle. A strong increase in the total number of emitted volatiles (TAGs) is visible in days 3,6 and 9 compared to day 0. The Venn diagram on the right highlights how volatiles (average percentage for all sites) were distributed during the time course of the experiment. Note that the sum of all values adds up to a little more than 100% because of rounding.

to identify tags (volatile compounds) that significantly differed in relative concentration among storage times. For further analysis, a volatile compound was considered to be present in a location when it was detected in at least two-thirds of the total number of samples (both technical and biological replicates) of that location.

Principal component analysis (PCA) was created with the Past software version 3.04 (Hammer et al., 2001) based on the relative concentration of tags that significantly varied in concentration in at least one site (Fig. 2A). PCA was generated based on the average values of three technical replicates and illustrates biological replicates (truffle fruiting bodies). The heatmap in Fig. 4 was produced based on the relative concentration of tags which significantly varied in concentration in three or all four sites. For the heatmap, tag concentrations in each row were divided by the maximum of the row to generate relative concentrations between zero and one.

2.3. Identification of volatile compounds

Volatile compounds were tentatively identified using mass spectra databases (National Institute of Standards and Technology (NIST) library v. 2.0, Gaithersburg, USA) and Kovats retention indices (calculated based on n-alkanes). Complete identification of volatile compounds was achieved using authentic standards for the following compounds purchased from Merck/Sigma-Aldrich (Darmstadt, Germany): propan-2-one (= acetone), (methylsulfanyl)methane (= DMS), butan-2-one, ethyl acetate, 2-methylpropan-1-ol, 3-methylbutanal, 2-methylbutanal, 2-methylbutan-1-ol, dimethyl sulfone, benzaldehyde, oct-1-en-3-ol, 2-phenylacetaldehyde, 2-phenylethan-1-ol.

2.4. DNA extraction and characterization of bacterial composition of T. aestivum fruiting bodies upon storage

DNA was isolated from truffle's gleba using DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. One sample of 100 ± 5 mg was extracted from each fruiting body subsamples at each time point. Quality and concentration of extracted DNA was examined by both spectrometry method (NanoDrop, Thermo Fisher) and gel electrophoresis. Microbial characterization was performed using PCR-high throughput amplicon sequencing. Amplicon libraries of 16S rRNA were produced using 787r (5′-ATTAGATACCYTGTAGTCC- 3′) (Nadkarni et al., 2002) and 1073f (5′-ACGAGCTGACGACARCCATG -3′) primers (On et al., 1998). Each primer contained a linker and a barcode which were used for the sample identification. Polymerase chain reactions (PCRs) were performed in a final volume of 25 µl containing 2 µl of template DNA, 10 µl of PCR Mastermix (5 PRIME) and 1 µl of each forward and reverse primers (0.2 µM). Amplification conditions were 94 °C for 10 min, 29 cycles 94 °C for 30 s, 48 °C for 45 s, 72 °C for 90 s, followed by 72 °C for 10 min. The concentration of PCR products was estimated by gel electrophoresis and 50 µl of each amplicon was sent for MiSeq Illumina sequencing at GeT PLAGE sequencing platform (INRA Toulouse).

Obtained sequences from amplicon sequencing were analyzed using FROGS (Find Rapidly OTU with Galaxy Solution) (Escudié et al., 2017) on the MIGALE Galaxy web platform (Afgan et al., 2018) by following processes: quality control of sequencing (quality score ≥ 30), demultiplexing (attributing each sequence to a sample), and pre-processing. Pre-processing consisted of removal of primers from sequences, sequences with insufficient primers, with ambiguous bases, out of the expected nucleotide length and identical sequences (dereplication). Clustering of the remaining sequences into operational taxonomic units (OTU) were conducted base on iterative Swarm algorithm. Chimaeras, singletons and rare OTUs (≤5 sequences in all samples) were excluded for further analyses. Clusters were affiliated to one taxonomy by blasting OTUs against SILVA database (Quast et al., 2012) and the ribosomal database project (RDP) classifier (Cole et al., 2009). OTUs bootstrap affiliation values of < 1 at phylum level or present in higher abundance in the negative control were removed from the data. Moreover, OTUs with bootstrap affiliation values of < 0.7 in other taxonomic ranks below phylum, were considered as unidentified. Remaining OTUs were rarefied (adjusting sequences randomly to the total abundance in the smallest sample) to 21,880 using Phyloseq package in R (McMurdie and Holmes, 2013). The raw data are deposited in the NCBI Sequence Read Archive website (http://www.ncbi.nlm.nih.gov/sra) under the BioProject study accession number PRJNA523325.

A PCA showing the evolution during storage of the bacterial community at the class level (Fig. 2B) was generated using Past software version 3.04 (Hammer et al., 2001). The bar chart in Fig. 3 representing changes in the nine dominant genera during storage was produced from the same matrix using Phyloseq package in R.

2.5. Quantification of the bacterial population within T. aestivum fruiting bodies upon storage

Total DNA was used to quantify the total bacterial 16S using the 16S rRNA gene-specific primers [10 µM each; 968F/1401R (total bacteria (Felske et al., 1998);). The DNA samples were first adjusted at the same concentration [5 ng/µl] after Nanodrop-1000 spectrometer (NanoDrop Technologies, Wilmington, DE, USA) analysis. Absolute quantifications

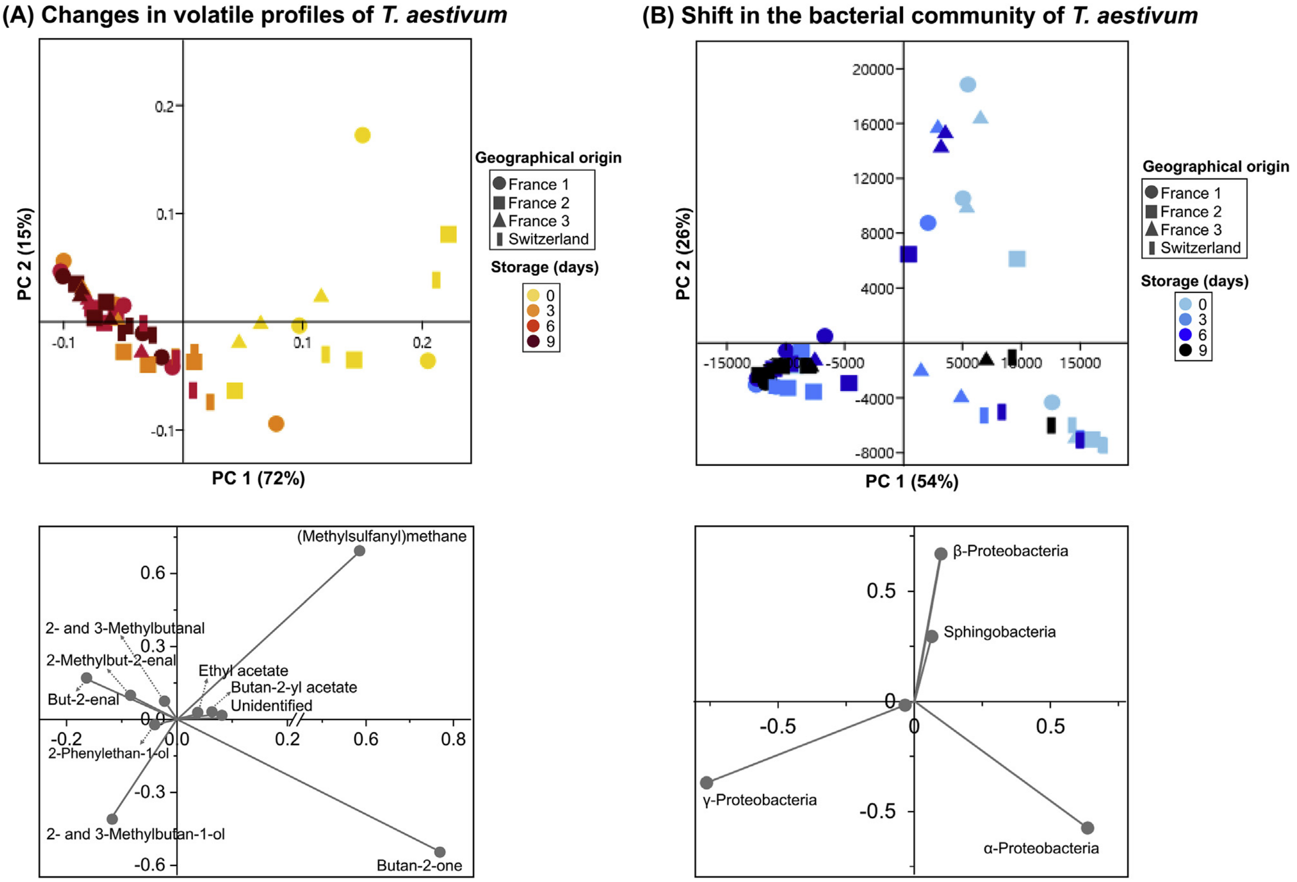

Fig. 2. Changes in volatile profiles and in the bacterial community of *T. aestivum* fruiting bodies during storage. (A) PCA based on volatiles which concentrations significantly varied during storage in truffles from at least one geographical origin ($p < 0.05$, Kruskal-Wallis test with $\alpha = 0.05$). Each point represents one truffle fruiting body (average value of three technical replicates). The loading plot below highlights the top twelve volatiles driving the PCA. (B) PCA and loading plot illustrating differences in microbial communities among samples and bacterial classes driving those differences. The loading plot below highlights the top five bacterial classes driving the PCA.

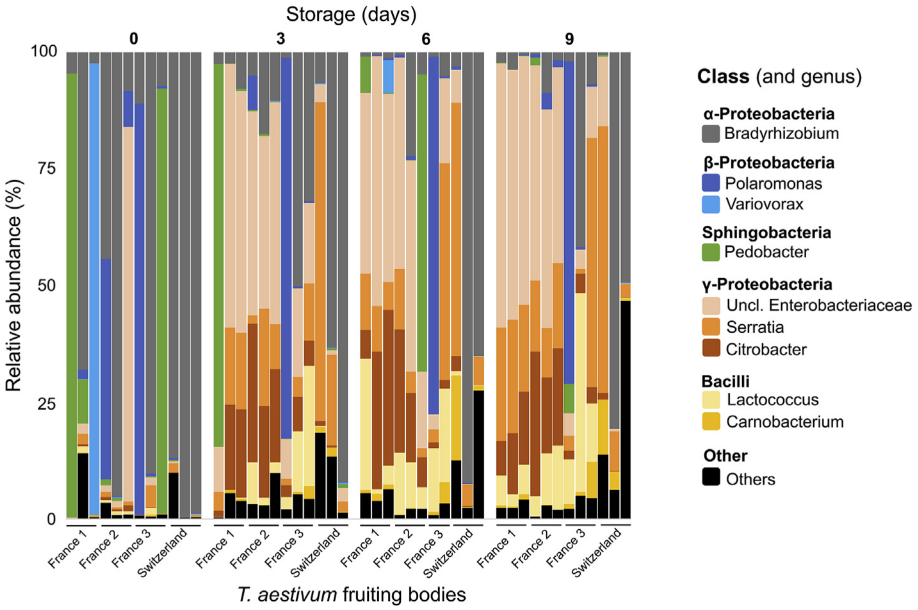

Fig. 3. Evolution of the truffle microbiome during storage. Changes in the relative proportion of the nine most abundant bacterial genera within single *T. aestivum* fruiting bodies. Each bar represents a single fruiting body.

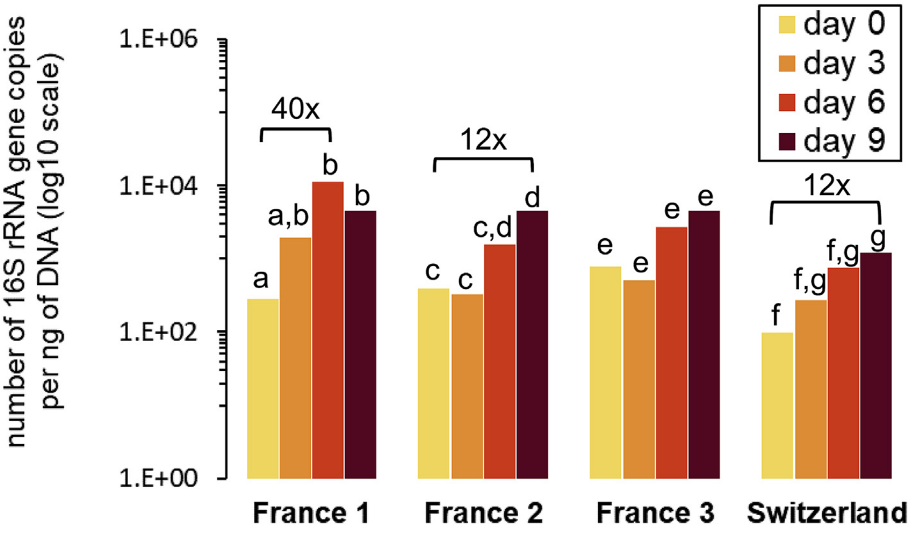

Fig. 4. Evolution of bacterial density during storage. Changes in bacterial cell density (number of 16S rRNA gene copies normalized to ng of fungal DNA) in *T. aestivum* fruiting bodies during storage. Bars represent the average values of three fruiting bodies per location and time point (days 0,3,6 and 9) and are shown on a log10 scale. Time points of different locations were compared considering each site independently (using non log transformed values). Different letters above bars indicate statistically significant differences ($p < 0.05$, Kruskal-Wallis test followed by the Dunn post-hoc test for multiple comparisons). Fold increase (based on the non-log transformed data) is indicated between day 0 and other time points where a significant change in bacterial cell density was detected.

were performed using serial dilutions of standard plasmids containing total bacterial 16S rDNA inserts (from 10^9 to 10^2 gene copies/μl) and the SsoAdvanced Universal SYBR Probes Supermix (classical qPCR for quantification of total bacteria) from Bio-Rad. The total bacterial quantifications were performed using the following cycle parameters: 1 cycle of 98 °C for 3 min followed by 40 cycles of 98 °C for 15 s, 56 °C for 30 s (AT: 56 °C. For each qPCR run using SYBR technology, a melting curve was performed at the end. Bacterial cell density within truffle gleba was expressed as number of 16S rRNA gene copies normalized to ng of fungal DNA. Bacterial cell density was compared among different time points considering each geographical location separately and using the Kruskal-Wallis and Dunn post-hoc test ($p < 0.05$).

2.6. *Linking volatile profiles to bacterial community structure by correlation network analysis*

To investigate the correlation between the evolution of bacterial composition and change in volatile compounds during storage, correlation network analysis was used. A linear correlation between significant volatile compounds present in at least one cites and the most abundant bacterial classes in the fruiting bodies were performed using the CORREL function in Excel. Network in Fig. 6 was generated by Cytoscape software (Shannon, 2003) with nodes representing volatile compounds and bacterial class and edges colour and thickness represent correlation coefficient. For the bacterial classes, nodes are size coded to reflect their relative abundance in the samples.

3. Results

3.1. *The aroma and microbial community of T. aestivum undergo deep changes during storage*

At first, we aimed at investigating the effect of storage on the volatile profile of the black truffle *T. aestivum*. Volatile profiling, performed on truffles from four geographical locations, generated a data matrix of 3,978 mass tags, where each tag corresponds to a specific mass fragment (m/z) in a specific time window. Comparing the number of tags per sites and during the aging process revealed a stark increase in the number of volatiles between day 0 (365 ± 58 (STE) tags) and the later time points (day 3: 1,207 ± 307 tags, day 6: 1,198 ± 181

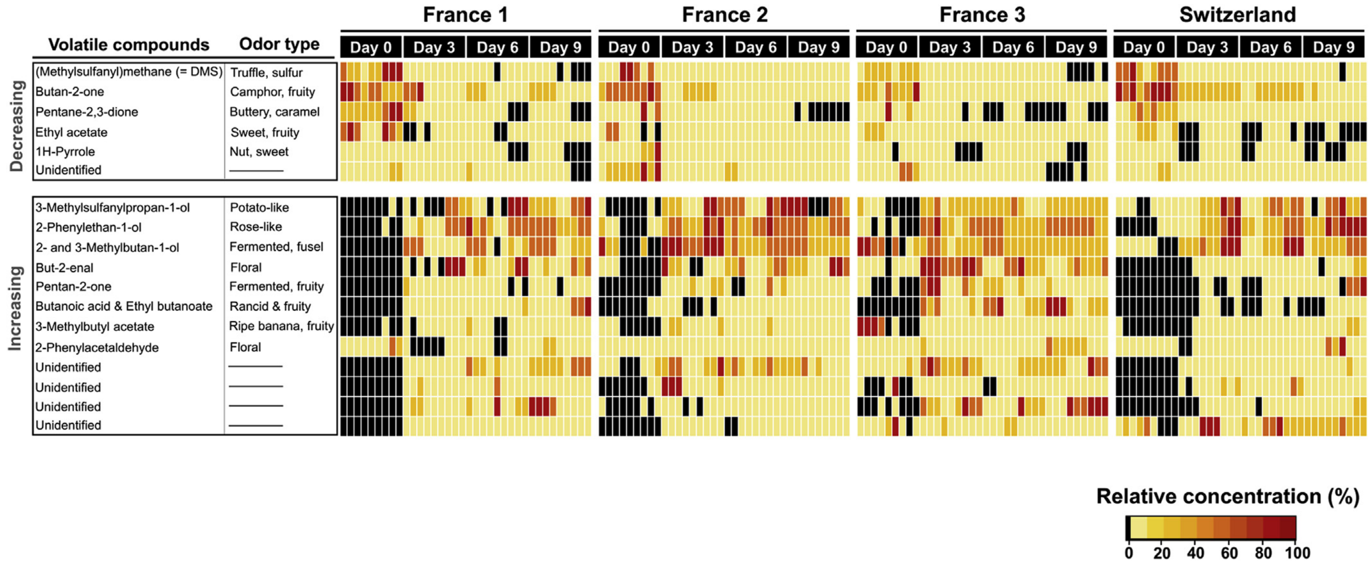

Fig. 5. Evolution of the volatile profile of truffles during storage. The heatmap illustrates volatiles consistently emitted by truffles in at least three or all the four geographical regions, and whose concentrations significantly decreased or increased with time ("freshness" and "spoilage marker", respectively) (n = 3 replicates per fruiting body, $p < 0.05$, Kruskal-Wallis test with $\alpha = 0.05$).

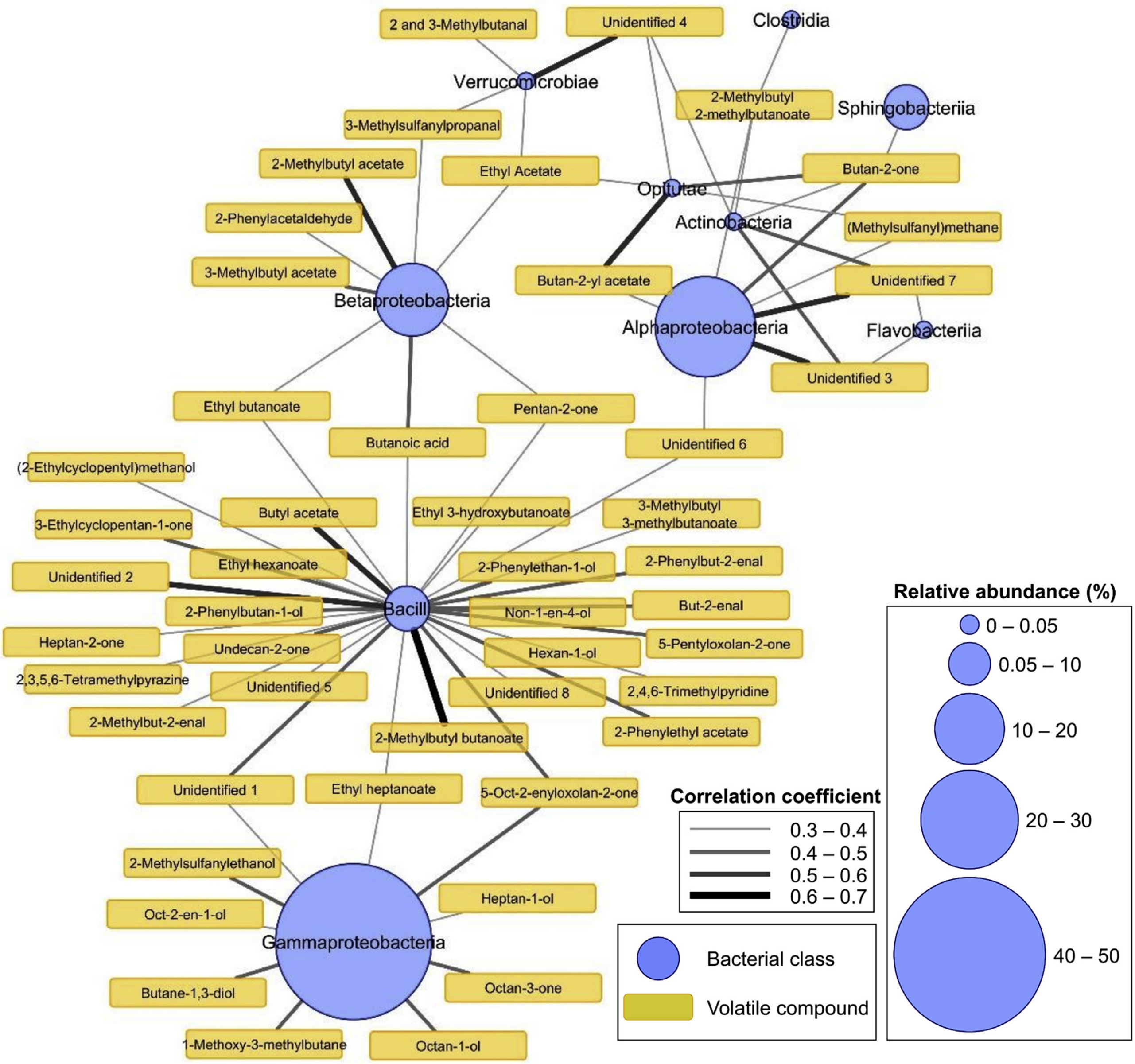

Fig. 6. Correlation network among bacteria and volatiles. Correlation between volatile compounds and bacterial communities of *T. aestivum*. Nodes represent most abundant bacterial classes and volatile compounds which significantly differed in concentration among storage times at least in one geographical location ($n = 3$ replicates per fruiting body, $p < 0.05$, Kruskal-Wallis test with $\alpha = 0.05$). In the case of bacterial classes, nodes are size coded to reflect their relative abundance. Edge's colour and thickness illustrate various correlation coefficient. Volatile compounds which a correlation coefficient < 0.3 are not shown (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

tags; day 9: 1,442 ± 66 tags) (Fig. 1). A Venn diagram was furthermore constructed to reflect the volatile profiles at each time point and considering all sites together (Fig. 1). It illustrates for instance that 13% (relative percentage of tags) of all volatiles were detectable at each time point and also highlights the marked increase in the proportion of time-point specific volatiles observed between day 0 (4%) and the other time points (day 3: 17%, day 6: 9%, day 9: 14%). Statistically comparing the relative concentrations of volatiles at each time point and separately for each site revealed that overall 43% of all volatiles (1,701 out of 3,968 tags) were significantly ($p < 0.05$, Kruskal-Wallis test) affected in at least one site during the time course of the experiment.

Our second aim was to identify the structure of volatiles, specifically focusing on those that changed in concentration during storage. To this end, the data matrix of 3,978 tags was filtered by keeping tags that occurred in at least two third of the truffles per site and that significantly varied in concentration in at least one site. A final filtering step was then applied to keep a single tag per volatile leading to a final matrix containing 71 volatiles (Table S1). PCA applied to this reduced matrix exemplified the important shift in volatile compounds emitted during storage (Fig. 2A). The PCA model explained 87% of the variance in the data while its loading plot illustrates the twelve major volatiles driving the shift observed in the volatile profile from day 0 to day 9 (Fig. 2A). Fresh truffles (day 0) contained more of the sulfur compound (methylsulfanyl)methane (synonym dimethyl sulfide (DMS)), the ketone butan-2-one and to a lesser extent, two esters (ethyl acetate and butan-2-yl acetate) compared to older (day 3,6 and 9) samples. By contrast, stored samples (days 3, 6, and 9) contained higher concentrations of aldehydes and alcohols including 2- and 3-methylbutanal, but-2-enal, 2-methylbut-2-enal, 2- and 3-methylbutan-1-ol and 2-phenylethan-1-ol (Fig. 2A).

Changes in bacterial community composition upon storage were characterized by 16S rDNA Illumina Miseq high throughput amplicon sequencing which resulted in 1,759,994 raw sequences. A total of 1,677,202 sequences remained after removal of low-quality sequences, chimaeras, and singletons with an average of 35,685 sequences per fruiting body throughout all locations. Sequences were clustered into 217 Operational Taxonomic Units (OTUs). Removal of potential contaminants and subsequent rarefaction of data to 21,880 reads per sample resulted in 195 OTUs to be further analyzed. A PCA (Fig. 2B) based on bacterial classes was created and explained 80% of the total variance based on the first two axes. The loading plot displays the most abundant bacterial classes in the truffle fruiting bodies during storage. Fresh truffles (day 0), were dominated by either α- and β-*Proteobacteria* or *Sphingobacteria*, whereas γ-*Proteobacteria,* and to a lesser extent *Bacilli* prevailed in most stored samples (day 3, 6, and 9). Some notable exceptions (i.e. some Swiss and French samples) were nevertheless visible and are discussed in more detail in the following section. Additionally, the PCA highlights that bacterial communities converge towards γ-*Proteobacteria* with time despite the initial diversity of dominant bacterial classes (α- and β-*Proteobacteria* or *Sphingobacteria*) at day 0 and regardless of the geographical origin of the truffles (Fig. 2B). Overall, our data demonstrate that storage caused enormous changes in aroma and bacterial community of *T. aestivum*, and most microbiomes/volatilomes converged to a similar endpoint regardless of different initial conditions.

3.2. *The native bacterial community of T. aestivum is replaced within a few days by food-spoilage bacteria*

Having observed the massive shift in the dominant bacterial classes upon storage, we subsequently assessed the changes in bacterial community structure within single fruiting bodies (Fig. 3). The most abundant genera in the fresh truffles (day 0) were *Bradyrhizobium* (α-*Proteobacteria* class) representing 51 ± 12% (average percentage based on the number of reads ± standard error) of the total community, followed by *Pedobacter* (*Sphingobacteria*) with 17 ± 10% and *Polaromonas* (β-*Proteobacteria*) with 12 ± 8% and Variovorax (β-*Proteobacteria*) with 8 ± 8%. Those genera were replaced in most cases and as early as day 3 by an unknown genus of the *Enterobacteriaceae* family*, Serratia and Citrobacter* genera (all γ-*Proteobacteria*) as well as by lactic acid bacteria (*Carnobacterium* and *Lactococcus* genera belonging to the Bacilli class). Reads corresponding to spoilage bacteria were already present at day 0 but in low abundance compared to other genera (0.9 ± 0.4% for the *Serratia*, 7 ± 7% for the *Enterobacteriaceae* genera and below 0.5% for the genera of lactic acid bacteria) and gradually increased up to 9 days of storage (28 ± 7% for the *Enterobacteriaceae* genera, 20 ± 5% for the *Serratia,* 10 ± 3% for Citrobacter, 9 ± 3% for *Lactococcus* and 2 ± 1% *Carnobacterium*). Three truffles nevertheless displayed a relatively stable bacterial community during aging and included one truffle from France (predominantly colonized by *Polaromonas* (β-*Proteobacteria*)) and two Swiss truffles, where *Bradyrhizobium* (α-*Proteobacteria*) was still dominant after 9 days of storage.

Changes during storage were not only noticeable in bacterial community composition, but also in terms of bacterial count (measured by quantifying 16S rRNA copies by qPCR). The average quantity of bacteria colonizing fresh truffle fruiting bodies from France and Switzerland varied from approximately 130 to 118 thousand [16S rRNA copies/ng of DNA] and mean bacterial density increased 12–40 times during storage within truffles originating from a single location, (Fig. 4). The data overall illustrate that storage lead to an increase in bacterial cell density within truffle fruiting bodies and to the dominance of food-spoilage bacteria.

3.3. *T. aestivuḿs freshness and spoilage markers*

Having demonstrated a shift in the volatile profiles of truffles during storage, we aimed at identifying specific freshness and spoilage markers. Out of the 71 volatiles of Table S1, most (89%) increased in concentration in at least one site compared to the initial time point of the experiment while only a small proportion (11%) decreased in concentration. The heatmap in Fig. 5 illustrates a subset of this data and shows 18 volatiles that followed the same trend in at least three of the four sites. Those volatiles can hence be considered as general freshness or spoilage markers. Freshness volatile markers (compounds which concentration decreased upon storage) included DMS, butan-2-one, 1H-pyrrole, and ethyl acetate as well as pentane-2,3-dione as seen in Fig. 5. By contrast, spoilage markers (compounds which concentration increased upon storage) comprised for instance 3-methylsulfanylpropan-1-ol, 2-phenylethan-1-ol and 2-phenylacetaldehyde, 2 and 3-methylbutan-1-ol, but-2-enal, along with butanoic acid and ethyl butanoate (Fig. 5).

3.4. *Changes in volatile profiles upon storage correlate to the dynamic in the bacterial community*

Considering the important shifts observed during storage in both volatile profiles and bacterial communities, we questioned to which extent these changes were linked to each other. A correlation network analysis was performed among single volatile compounds and bacterial classes. Specifically, the most abundant bacterial classes (with a relative abundance of more than 8%) and volatile compounds which concentrations significantly varied upon storage in at least one location were included in this analysis (Fig. 6). The network illustrates both qualitative and quantitative differences in a way that bacteria and volatile compounds are connected. For instance, freshness markers (i.e. ethyl acetate, dimethyl sulfide (syn. (methylsulfanyl)methane), and butan-2-one) are linked to many bacterial classes that also include some of the classes typical of fresh truffles (*α*- and *β*-*Proteobacteria* or *Sphingobacteria*). By contrast, *Bacilli* (*i.e. Lactococcus, Carnobacterium* genera in Fig. 3) and γ-*Proteobacteria* that increased in abundance during storage (Figs. 2B and 3) were linked to many different volatile compounds. Specifically, the abundance of *Bacilli* bacteria correlated with numerous alcohols, ketones and aldehydes (i.e. four carbon-containing volatiles (C_4 compounds): ethyl butanoate, and 2-methylbutyl butanoate), as well as aromatic compounds (i.e. 2-phenylethanol, and 2-phenylbut-2-enal), and some other compounds (pyridine and pyrazine, Fig. 6). γ-*Proteobacteria* were correlated with eight carbon-containing volatiles such as octan-3-one, oct-2-en-1-ol and octan-1-ol. Overall, our data suggest a potential link among distinct bacterial classes and specific volatile compounds.

4. Discussion

Truffle fungi are highly perishable culinary delicacies that spoil within a few weeks from harvest and lose their aroma due to dehydration and microbial growth (Nazzaro et al., 2007; Rivera et al., 2011b, 2011a; 2010b; Saltarelli et al., 2008). In this study, we investigated how storage influenced endemic microbial communities within truffles and related this to changes in aroma profiles.

4.1. *Food spoilage bacteria gradually replace the endemic truffle microbiome upon storage*

Our results evidenced that most fresh truffles were predominantly colonized by bacteria of α-*Proteobacteria* class (*Bradyrhizobium* genus). The microbiome of a smaller portion of fruiting bodies (about 25%) were nevertheless dominated by members of the β-*Proteobacteria* (*Polaromonas* genus) or *Sphingobacteria* (*Pedobacter* genus) classes. These results corroborate recent findings on the same species (Splivallo et al., unpublished results) as well as earlier findings describing the importance of bacteria of the α-*Proteobacteria* class in various truffle species (Antony-Babu et al., 2014; Benucci and Bonito, 2016; Splivallo et al., unpublished results; Vahdatzadeh et al., 2015; Ye et al., 2018). The increase in bacterial population of 4–30 times during storage observed here similarly mirrors earlier results obtained by culture dependent methods (Saltarelli et al., 2008). A gradual replacement of the endemic microbiome by members of the γ-*Proteobacteria* class (i.e. *Enterobacteriaceae* family) and to a lesser extent by members of the *Bacilli* class (different families belonging to the *Lactic Acid Bacteria* order) was also evidenced by our data. The *Enterobacteriaceae* family include several food-borne pathogens that have been described earlier in truffles (Nazzaro et al., 2007; Reale et al., 2009; Rivera et al., 2010a, 2010b) but also in many other foods (Blackburn, 2006; Lim et al., 2014). Specifically, members of the *Serratia* genus that appeared already after three days of storage include some known human pathogens (Mahlen, 2011) as well as food spoilage agents (i.e. causing off-note in meats and clotting milk products (Blackburn, 2006; Hernández-Macedo et al., 2011)). *Lactic acid bacteria* (genera of *Lactococcus* and *Carnobacterium*) similarly appearing after three days of storage have been described in *T. aestivum* as prevalent microorganisms involved in post-harvest spoilage (Reale et al., 2009; Rivera et al., 2011b, 2011a, 2010b). Attempt to delay spoilage by various post-harvest techniques have been applied to *T. aestivum* (Nazzaro et al., 2007; Reale et al., 2009; Rivera et al., 2011b; Saltarelli et al., 2008). For instance, combining modified atmosphere packaging with gamma-ray irradiation and refrigeration was shown to extend the shelf-life of *T. aestivum* to 21 days by reducing the population of cultivable food spoilage bacteria (Nazzaro et al., 2007). Overall, this indicates that further improving the shelf-life of truffles might be achievable by combining preservation techniques to simultaneously preserve the endemic truffle microbiome and limit the growth of spoilage microbes.

4.2. Changes in aroma profiles might explain quality loss upon storage

Our results revealed the existence of five freshness and twelve spoilage volatile markers in *T. aestivum*. Many of these volatiles are known to contribute to human-sensed truffle aroma (Culleré et al., 2010; Liu et al., 2012; Schmidberger and Schieberle, 2017; Splivallo and Ebeler, 2015). Identified freshness markers, volatiles that decreased in concentration during storage, included DMS, a compound with characteristic truffely and sulfurous notes (Culleré et al., 2010), butan-2-one (ethereal, camphor-like (Garg et al., 2018), pentane-2,3-dione (buttery, caramel-like (Schmidberger and Schieberle, 2017)), ethyl acetate (sweet, green (Garg et al., 2018)), and 1H-pyrrole (nutty, sweet (Büttner, 2017)). Some of the identified spoilage markers, volatiles that increased in concentration upon storage, included 2-phenylethan-1-ol (rose-like (Splivallo and Culleré, 2016)) and 2-phenylacetaldehyde (floral (Schmidberger and Schieberle, 2017)) and 2- and 3-methylbutan-1-ol (fermented, fusel (Schmidberger and Schieberle, 2017)).

Similar to what has been observed here, the concentrations of DMS and butan-2-one were shown to decrease during storage or post-harvest processing (i.e. gamma-ray irradiation, freeze-drying) in *T. melanosproum*, *T. aestivum* and *T. magnatum* (Aprea et al., 2007; Campo et al., 2017; Culleré et al., 2013, 2012; Palacios et al., 2014, 2014). This suggests that these volatiles might serve as freshness markers in numerous truffle species. Similarly, the spoilage markers 2- and 3-methylbutan-1-ol were reported to increase in concentrations in stored *T. melanosporum, T. magnatum* and *T. borchii* stored at 0 °C (Aprea et al., 2007; Bellesia et al., 2001, 1998), once again indicating that they might be considered as universal spoilage markers in truffles. In terms of human sensed aroma, stored samples of *T. aestivum* started developing strong off-flavours noticeable to the human nose after six storage days and had a dominant rotting smell after nine days (data not shown). It is reasonable to argue that the decrease in freshness markers and the appearance of spoilage markers were driving those changes in human sensed aroma. Changes in aroma perception can result from a shift in the proportion of odorants initially present in fresh samples or from the appearance of new (spoilage) odorants (Ridgway et al., 2010). For instance, an increase in the concentrations of pentan-2-one (fermented, fruity), and ethyl butanoate (fruity (Culleré et al., 2010)), two of our spoilage markers for *T. aestivum*, were reported to cause off-odours in refrigerated smoked salmon (Joffraud et al., 2001). Similarly, high concentrations of butanoic acid (rancid, cheesy note (Garg et al., 2018)), another spoilage marker in our study, has been shown to contribute to spoiled meat flavour (Ercolini et al., 2011; Jones, 2004).

Overall, our data suggest that the decrease in freshness markers and appearance of spoilage markers might be responsible for the change in aroma quality. Demonstrating the contribution of specific volatiles will however require the use of proper sensory techniques (i.e. GC-olfactometry) as well as the absolute quantification of odorants to determine odour activity values.

4.3. Specific microbes might produce particular volatiles during storage

Fresh truffles emit a blend of cyclical sulfur volatiles which are partially derived from bacteria as demonstrated in the specific case of *T. borchii* (Splivallo et al., 2015). We further speculated that many other volatile compounds typical of fresh truffle aroma could be partially derived from microbes inhabiting truffle fruiting bodies, overall putting forward the hypothesis of the mixed bacterial and fungal origin of truffle aroma (Murat et al., 2018; Splivallo et al., 2015, 2011; Vahdatzadeh et al., 2015). Indeed, both axenic cultures of truffles and a wide range of bacteria have the ability to emit numerous volatiles that make up fresh truffle aroma (Du et al., 2014; Lemfack et al., 2014; Li et al., 2012; Vahdatzadeh and Splivallo, 2018). The data presented here give further ground to that hypothesis since a strong correlation was observed between specific microbial classes and volatiles. For instance, the freshness markers DMS, butan-2-one, and ethyl acetate were correlated to bacterial classes typical of fresh truffles (*α*- and *β-Proteobacteria* or *Sphingobacteria*). Similarly, some spoilage markers (2-phenylethan-1-ol, but-2-enal, butanoic acid) were correlated to bacteria of the *Bacilli* class, which relative abundance increased during storage. Members of the *Bacilli* class have the ability to emit 2-phenylethan-1-ol and butanoic acid, as many other bacterial classes do (Lemfack et al., 2014). The strongest increase during storage was nevertheless observed in the relative abundance of γ-*Proteobacteria,* which correlated to the concentrations of three "eight carbon atoms" containing volatiles (octan-3-one, octan-1-ol and oct-2-en-1-ol). Other "C_8 volatiles" (i.e. octan-3-one and oct-1-en-3-ol) are predominantly emitted by fungi, including truffles (Lemfack et al., 2014; Splivallo et al., 2011). The production of those volatiles might be induced by bacteria as recently observed in the fungus *Mortierella elongate* (Uehling et al., 2017), suggesting that a similar scenario might happen in truffles.

The notion that the microbiome of truffles fully produces truffle aroma is tempting but one should be reminded that correlation does not always imply causation. Indeed, three samples (25% of all samples) of this study presented relatively resilient bacterial communities up to day 9 of storage, which was however not observable in terms of the volatilome of the same samples. These differences might be explained by unusually heterogeneous samples in terms of microbiomes or by the possibility that the volatilome of *T. aestivum* is predominantly derived from truffles and not from bacteria. Even though supported by a contrasting number of observations, the latter two hypotheses (bacteria or truffle do most of the aroma) nevertheless begs for further experiments to tell apart and resolve the bacterial and fungal contribution to truffle aroma. Last, spoilage fungi might have also contributed to some of the changes in aroma reported here and should hence be monitored in further studies for providing a more holistic picture of the spoilage process.

5. Conclusion

Altogether, our data highlight the dynamic and the deep changes that truffle aroma and microbiome undergo during storage. Our findings also suggest that commensal and spoilage microbes might be directly or indirectly driving the shift in aroma profile observed upon aging, and hence pave the way towards new preservation techniques.

Funding

The data generated on the truffle microbiome was supported by the Laboratory of Excellence ARBRE ANR-11-LABX 0002 01 - INRA and the financial contribution of the PEPS Mirabelle (project Truffinet) Université de Lorraine and CNRS.

Research funding was provided for MV and RS through the LOEWE research program of the government of Hesse (IPF), Germany, in the framework of the Integrative Fungal Research Cluster (IPF) and by the German Research Foundation/Deutsche Forschungsgemeinschaft (DFG) – grant number 1191/5-1.

Authors contributions

MV and RS conceived the experiments and MV performed all experimental procedures. Microbiome sequencing was done by MV and AD. AD quantified bacterial DNA in truffle samples. Data analysis and statistics were performed by MV with input from AD and RS. MV and RS wrote the manuscript with input from AD.

Declarations of interest

None.

Acknowledgements

We are grateful to the INRA MIGALE bioinformatics platform (http://migale.jouy.inra.fr) for providing help and support. The authors thanks Dr. A. Cébron (LIEC laboratory) for providing the qPCR protocol and L. Fauchery and M. Maaroufi from the Ecogenomic platform for technical assistance on the 16SrRNA qPCR analysis. The authors are also thankful to Dr. G. Chevalier for providing the truffle samples from France.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.103251.

References

Afgan, E., Baker, D., Batut, B., van den Beek, M., Bouvier, D., Čech, M., Chilton, J., Clements, D., Coraor, N., Grüning, B.A., Guerler, A., Hillman-Jackson, J., Hiltemann, S., Jalili, V., Rasche, H., Soranzo, N., Goecks, J., Taylor, J., Nekrutenko, A., Blankenberg, D., 2018. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. Nucleic Acids Res. 46, W537–W544. https://doi.org/10.1093/nar/gky379.

Antony-Babu, S., Deveau, A., Van Nostrand, J.D., Zhou, J., Le Tacon, F., Robin, C., Frey-Klett, P., Uroz, S., 2014. Black truffle associated bacterial communities during the development and maturation of *Tuber melanosporum* ascocarps and putative functional roles: *Tuber melanosporum* associated bacterial communities. Environ. Microbiol. 16, 2831–2847. https://doi.org/10.1111/1462-2920.12294.

Aprea, E., Biasioli, F., Carlin, S., Versini, G., Märk, T.D., Gasperi, F., 2007. Rapid white truffle headspace analysis by proton transfer reaction mass spectrometry and comparison with solid-phase microextraction coupled with gas chromatography/mass spectrometry. Rapid Commun. Mass Spectrom. 21, 2564–2572. https://doi.org/10.1002/rcm.3118.

Barbieri, E., Bertini, L., Rossi, I., Ceccaroli, P., Saltarelli, R., Guidi, C., Zambonelli, A., Stocchi, V., 2005. New evidence for bacterial diversity in the ascoma of the ectomycorrhizal fungus *Tuber borchii* Vittad. FEMS Microbiol. Lett. 247, 23–35. https://doi.org/10.1016/j.femsle.2005.04.027.

Barbieri, E., Guidi, C., Bertaux, J., Frey-Klett, P., Garbaye, J., Ceccaroli, P., Saltarelli, R., Zambonelli, A., Stocchi, V., 2007. Occurrence and diversity of bacterial communities in *Tuber magnatum* during truffle maturation. Environ. Microbiol. 9, 2234–2246. https://doi.org/10.1111/j.1462-2920.2007.01338.x.

Bellesia, F., Pinetti, A., Bianchi, A., Tirillini, B., 1998. The volatile organic compounds of black truffle (*Tuber melanosporum Vitt.*) from Middle Italy. Flavour Fragrance J. 13, 56–58. https://doi.org/10.1002/(SICI)1099-1026(199801/02)13:1 < 56::AID-FFJ692 > 3.0.CO;2-X.

Bellesia, F., Pinetti, A., Tirillini, B., Bianchi, A., 2001. Temperature-dependent evolution of volatile organic compounds in *Tuber borchii* from Italy. Flavour Fragrance J. 16, 1–6. https://doi.org/10.1002/1099-1026(200101/02)16:1 < 1::AID-FFJ936 > 3.0.CO;2-Y.

Benucci, G.M.N., Bonito, G.M., 2016. The truffle microbiome: Species and geography effects on bacteria associated with fruiting bodies of hypogeous *Pezizales*. Microb. Ecol. 72, 4–8. https://doi.org/10.1007/s00248-016-0755-3.

Blackburn, C. de W., 2006. Food Spoilage Microorganisms, first ed. CRC Press, Woodhead Publishing Limited, Cambridge, England.

Büttner, A. (Ed.), 2017. Springer Handbook of Odor. Springer, Cham.

Campo, E., Marco, P., Oria, R., Blanco, D., Venturini, M.E., 2017. What is the best method for preserving the genuine black truffle (*Tuber melanosporum*) aroma? An olfactometric and sensory approach. Lebensm. Wiss. Technol. 80, 84–91. https://doi.org/10.1016/j.lwt.2017.02.009.

Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., et al., 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res. 37, D141–D145. https://doi.org/10.1093/nar/gkn879.

Culleré, L., Ferreira, V., Chevret, B., Venturini, M.E., Sánchez-Gimeno, A.C., Blanco, D., 2010. Characterisation of aroma active compounds in black truffles (*Tuber melanosporum*) and summer truffles (*Tuber aestivum*) by gas chromatography–olfactometry. Food Chem. 122, 300–306. https://doi.org/10.1016/j.foodchem.2010.02.024.

Culleré, L., Ferreira, V., Venturini, M.E., Marco, P., Blanco, D., 2013. Chemical and sensory effects of the freezing process on the aroma profile of black truffles (*Tuber melanosporum*). Food Chem. 136, 518–525. https://doi.org/10.1016/j.foodchem.2012.08.030.

Culleré, L., Ferreira, V., Venturini, M.E., Marco, P., Blanco, D., 2012. Evaluation of gamma and electron-beam irradiation on the aromatic profile of black truffle (*Tuber melanosporum*) and summer truffle (*Tuber aestivum*). Innov. Food Sci. Emerg. Technol. 13, 151–157. https://doi.org/10.1016/j.ifset.2011.09.003.

Du, M., Huang, S., Wang, J., 2014. The volatiles from fermentation product of *Tuber formosanum*. Open J. For. 04, 426–429. https://doi.org/10.4236/ojf.2014.44047.

Dunkel, A., Steinhaus, M., Kotthoff, M., Nowak, B., Krautwurst, D., Schieberle, P., Hofmann, T., 2014. Nature's chemical signatures in human olfaction: A foodborne perspective for future biotechnology. Angew. Chem. Int. Ed. 53, 7124–7143. https://doi.org/10.1002/anie.201309508.

Ercolini, D., Ferrocino, I., Nasi, A., Ndagijimana, M., Vernocchi, P., La Storia, A., Laghi, L., Mauriello, G., Guerzoni, M.E., Villani, F., 2011. Monitoring of microbial metabolites and bacterial diversity in beef stored under different packaging conditions. Appl. Environ. Microbiol. 77, 7372–7381. https://doi.org/10.1128/AEM.05521-11.

Escudié, F., Auer, L., Bernard, M., Mariadassou, M., Cauquil, L., Vidal, K., Maman, S., Hernandez-Raquet, G., Combes, S., Pascal, G., 2017. FROGS: Find, rapidly, OTUs with galaxy solution. Bioinformatics. https://doi.org/10.1093/bioinformatics/btx791.

Falasconi, M., Pardo, M., Sberveglieri, G., Battistutta, F., Piloni, M., Zironi, R., 2005. Study of white truffle aging with SPME-GC-MS and the Pico2-electronic nose. Sensor. Actuator. B Chem. 106, 88–94. https://doi.org/10.1016/j.snb.2004.05.041.

Fassi, B., Fontana, A., 1967. Sintesi micorrizica tra Pinus strobus e *Tuber maculatum I.* Micorrize e sviluppo dei semenzali nel secondo anno. Allionia 13, 177–186 Allionia.

Felske, A., Akkermans, A.D., De Vos, W.M., 1998. Quantification of 16S rRNAs in complex bacterial communities by multiple competitive reverse transcription-PCR in temperature gradient gel electrophoresis fingerprints. Appl. Environ. Microbiol. 64, 4581–4587.

Fiecchi, A., Kienle, M., Scala, A., Cabella, P., 1967. Bis-methylthiomethane, an odorous substance from white truffle, *Tuber magnatum Pico*. Tetrahedron Lett. 8, 1681–1682.

Garg, N., Sethupathy, A., Tuwani, R., Nk, R., Dokania, S., Iyer, A., Gupta, A., Agrawal, S., Singh, N., Shukla, S., Kathuria, K., Badhwar, R., Kanji, R., Jain, A., Kaur, A., Nagpal, R., Bagler, G., 2018. FlavorDB: A database of flavor molecules. Nucleic Acids Res. 46, D1210–D1216. https://doi.org/10.1093/nar/gkx957.

Gentleman, R., Ihaka, R., 1996. A language for data analysis and graphics. Journal of computational and graphical statistics. J. Comput. Graph. Stat. 5, 299–314.

Gryndler, M., Soukupová, L., Hršelová, H., Gryndlerová, H., Borovička, J., Streiblová, E., Jansa, J., 2013. A quest for indigenous truffle helper prokaryotes: *Tuber aestivum* -associative prokaryotes. Environ. Microbiol. Rep. 5, 346–352. https://doi.org/10.1111/1758-2229.12014.

Hammer, Ø., Harper, D.A., Ryan, P.D., 2001. PAST: Paleontological statistics software: Package for education and data analysis. Palaeontol. Electron. 4, 9.

Hernández-Macedo, M.L., Barancelli, G.V., Contreras-Castillo, C.J., 2011. Microbial deterioration of vacuum-packaged chilled beef cuts and techniques for microbiota detection and characterization: A review. Braz. J. Microbiol. 42, 1–11. https://doi.org/10.1590/S1517-83822011000100001.

Joffraud, J.J., Leroi, F., Roy, C., Berdagué, J.L., 2001. Characterisation of volatile compounds produced by bacteria isolated from the spoilage flora of cold-smoked salmon. Int. J. Food Microbiol. 66, 175–184. https://doi.org/10.1016/S0168-1605(00)00532-8.

Jones, R.J., 2004. Observations on the succession dynamics of lactic acid bacteria populations in chill-stored vacuum-packaged beef. Int. J. Food Microbiol. 90, 273–282.

Lemfack, M.C., Nickel, J., Dunkel, M., Preissner, R., Piechulla, B., 2014. mVOC: A database of microbial volatiles. Nucleic Acids Res. 42, D744–D748. https://doi.org/10.1093/nar/gkt1250.

Li, Y.-Y., Wang, G., Li, H.-M., Zhong, J.-J., Tang, Y.-J., 2012. Volatile organic compounds from a *Tuber melanosporum* fermentation system. Food Chem. 135, 2628–2637. https://doi.org/10.1016/j.foodchem.2012.07.013.

Lim, J.-A., Lee, D.H., Heu, S., 2014. The interaction of human enteric pathogens with plants. Plant Pathol. J. 30, 109–116. https://doi.org/10.5423/PPJ.RW.04.2014.0036.

Liu, R.-S., Li, D.-C., Li, H.-M., Tang, Y.-J., 2012. Evaluation of aroma active compounds in *Tuber* fruiting bodies by gas chromatography–olfactometry in combination with aroma reconstitution and omission test. Appl. Microbiol. Biotechnol. 94, 353–363. https://doi.org/10.1007/s00253-011-3837-7.

Luedemann, A., von Malotky, L., Erban, A., Kopka, J., 2011. TagFinder: Preprocessing software for the fingerprinting and the profiling of gas chromatography–mass Spectrometry based metabolome analyses. In: Hardy, N.W., Hall, R.D. (Eds.), Plant Metabolomics. Humana Press, Totowa, NJ, pp. 255–286. https://doi.org/10.1007/978-1-61779-594-7_16.

Mahlen, S.D., 2011. Serratia infections: From military experiments to current practice. Clin. Microbiol. Rev. 24, 755–791. https://doi.org/10.1128/CMR.00017-11.

McMurdie, P.J., Holmes, S., 2013. Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8, e61217. https://doi.org/10.18129/B9.bioc.phyloseq.

Murat, C., Payen, T., Noel, B., Kuo, A., Morin, E., Chen, J., Kohler, A., Krizsán, K., Balestrini, R., Da Silva, C., Montanini, B., Hainaut, M., Levati, E., Barry, K.W., Belfiori, B., Cichocki, N., Clum, A., Dockter, R.B., Fauchery, L., Guy, J., Iotti, M., Le Tacon, F., Lindquist, E.A., Lipzen, A., Malagnac, F., Mello, A., Molinier, V., Miyauchi, S., Poulain, J., Riccioni, C., Rubini, A., Sitrit, Y., Splivallo, R., Traeger, S., Wang, M., Žifčáková, L., Wipf, D., Zambonelli, A., Paolocci, F., Nowrousian, M., Ottonello, S., Baldrian, P., Spatafora, J.W., Henrissat, B., Nagy, L.G., Aury, J.-M., Wincker, P., Grigoriev, I.V., Bonfante, P., Martin, F.M., 2018. *Pezizomycetes* genomes reveal the molecular basis of ectomycorrhizal truffle lifestyle. Nat. Ecol. Evol. 2, 1956–1965. https://doi.org/10.1038/s41559-018-0710-4.

Nadkarni, M.A., Martin, F.E., Jacques, N.A., Hunter, N., 2002. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology 148, 257–266. https://doi.org/10.1099/00221287-148-1-257.

Nazzaro, F., Fratianni, F., Picariello, G., Coppola, R., Reale, A., Luccia, A.D., 2007. Evaluation of gamma rays influence on some biochemical and microbiological aspects in black truffles. Food Chem. 103, 344–354. https://doi.org/10.1016/j.foodchem.2006.07.067.

On, S.L.W., Atabay, H.I., Corry, J.E.L., Harrington, C.S., Vandamme, P., 1998. Emended description of Campylobacter sputorum and revision of its infrasubspecific (biovar) divisions, including C. sputorum biovar paraureolyticus, a urease-producing variant from cattle and humans. Int. J. Syst. Evol. Microbiol. 48, 195–206. https://doi.org/10.1099/00207713-48-1-195.

Palacios, I., Guillamón, E., García-Lafuente, A., Villares, A., 2014. Effects of freeze-drying treatment on the aromatic profile of *Tuber spp.* truffles: Effects of freeze-drying on the aroma of truffles. J. Food Process. Preserv. 38, 768–773. https://doi.org/10.1111/jfpp.12028.

Pennazza, G., Fanali, C., Santonico, M., Dugo, L., Cucchiarini, L., Dachà, M., D'Amico, A., Costa, R., Dugo, P., Mondello, L., 2013. Electronic nose and GC–MS analysis of volatile compounds in *Tuber magnatum Pico*: Evaluation of different storage conditions. Food Chem. 136, 668–674. https://doi.org/10.1016/j.foodchem.2012.08.086.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2012. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. Nucleic Acids Res. 41, D590–D596. https://doi.org/10.1093/nar/gks1219.

Reale, A., Sorrentino, E., Iacumin, L., Tremonte, P., Manzano, M., Maiuro, L., Comi, G., Coppola, R., Succi, M., 2009. Irradiation treatments to improve the shelf life of fresh black truffles (truffles preservation by gamma-rays). J. Food Sci. 74, M196–M200. https://doi.org/10.1111/j.1750-3841.2009.01142.x.

Ridgway, K., Lalljie, S.P.D., Smith, R.M., 2010. Analysis of food taints and off-flavours: A review. Food Addit. Contam. A 27, 146–168. https://doi.org/10.1080/19440040903296840.

Rivera, C.S., Blanco, D., Oria, R., Venturini, M.E., 2010a. Diversity of culturable microorganisms and occurrence of Listeria monocytogenes and Salmonella spp. in *Tuber aestivum* and *Tuber melanosporum* ascocarps. Food Microbiol. 27, 286–293. https://doi.org/10.1016/j.fm.2009.11.001.

Rivera, C.S., Blanco, D., Salvador, M.L., Venturini, M.E., 2010b. Shelf-life extension of fresh *Tuber aestivum* and *Tuber melanosporum* truffles by modified atmosphere packaging with microperforated films. J. Food Sci. 75, E225–E233. https://doi.org/10.1111/j.1750-3841.2010.01602.x.

Rivera, C.S., Venturini, M.E., Marco, P., Oria, R., Blanco, D., 2011a. Effects of electron-beam and gamma irradiation treatments on the microbial populations, respiratory activity and sensory characteristics of *Tuber melanosporum* truffles packaged under modified atmospheres. Food Microbiol. 28, 1252–1260. https://doi.org/10.1016/j.fm.2011.05.002.

Rivera, C.S., Venturini, M.E., Oria, R., Blanco, D., 2011b. Selection of a decontamination treatment for fresh *Tuber aestivum* and *Tuber melanosporum* truffles packaged in modified atmospheres. Food Control 22, 626–632. https://doi.org/10.1016/j.foodcont.2010.10.015.

Saltarelli, R., Ceccaroli, P., Cesari, P., Barbieri, E., Stocchi, V., 2008. Effect of storage on biochemical and microbiological parameters of edible truffle species. Food Chem. 109, 8–16. https://doi.org/10.1016/j.foodchem.2007.11.075.

Sbrana, C., Agnolucci, M., Bedini, S., Lepera, A., Toffanin, A., Giovannetti, M., Nuti, M.P., 2002. Diversity of culturable bacterial populations associated to *Tuber borchii* ectomycorrhizas and their activity on *T. borchii* mycelial growth. FEMS Microbiol. Lett. 211, 195–201. https://doi.org/10.1111/j.1574-6968.2002.tb11224.x.

Schmidberger, P.C., Schieberle, P., 2017. Characterization of the key aroma compounds in white Alba truffle (*Tuber magnatum pico*) and Burgundy truffle (*Tuber uncinatum*) by means of the sensomics approach. J. Agric. Food Chem. 65, 9287–9296. https://doi.org/10.1021/acs.jafc.7b04073.

Selosse, M.-A., Schneider-Maunoury, L., Taschen, E., Rousset, F., Richard, F., 2017. Black truffle, a hermaphrodite with forced unisexual behaviour. Trends Microbiol. 25, 784–787. https://doi.org/10.1016/j.tim.2017.05.010.

Shannon, P., 2003. Cytoscape: A software environment for integrated models of biomolecular interaction networks. Genome Res. 13, 2498–2504. https://doi.org/10.1101/gr.1239303.

Splivallo, R., Culleré, L., 2016. The smell of truffles: From aroma biosynthesis to product quality. In: Zambonelli, Alessandra, Iotti, M., Murat, C. (Eds.), True Truffle (*Tuber Spp.*) in the World. Springer International Publishing, Cham, pp. 393–407. https://doi.org/10.1007/978-3-319-31436-5_23.

Splivallo, R., Deveau, A., Valdez, N., Kirchhoff, N., Frey-Klett, P., Karlovsky, P., 2015. Bacteria associated with truffle-fruiting bodies contribute to truffle aroma: Bacteria produce truffle volatiles. Environ. Microbiol. 17, 2647–2660. https://doi.org/10.1111/1462-2920.12521.

Splivallo, R., Ebeler, S.E., 2015. Sulfur volatiles of microbial origin are key contributors to human-sensed truffle aroma. Appl. Microbiol. Biotechnol. 99, 2583–2592. https://doi.org/10.1007/s00253-014-6360-9.

Splivallo, R., Ottonello, S., Mello, A., Karlovsky, P., 2011. Truffle volatiles: From chemical ecology to aroma biosynthesis. New Phytol. 189, 688–699.

Splivallo, R., Valdez, N., Kirchhoff, N., Ona, M.C., Schmidt, J.-P., Feussner, I., Karlovsky, P., 2012. Intraspecific genotypic variability determines concentrations of key truffle volatiles. New Phytol. 194, 823–835. https://doi.org/10.1111/j.1469-8137.2012.04077.x.

Splivallo, R., Vahdatzadeh, M., Maciá-Vicente, J.G., Molinier, V., Peter, M., Egli, S., Uroz, S., Paolocci, F., Deveau, A., 2019. Orchard conditions and fruiting body characteristics drive the microbiome of the black truffle Tuber aestivum. Front. Microbiol. 10. https://doi.org/10.3389/fmicb.2019.01437.

Uehling, J., Gryganskyi, A., Hameed, K., Tschaplinski, T., Misztal, P.K., Wu, S., Desirò, A., Vande Pol, N., Du, Z., Zienkiewicz, A., Zienkiewicz, K., Morin, E., Tisserant, E., Splivallo, R., Hainaut, M., Henrissat, B., Ohm, R., Kuo, A., Yan, J., Lipzen, A., Nolan, M., LaButti, K., Barry, K., Goldstein, A.H., Labbé, J., Schadt, C., Tuskan, G., Grigoriev, I., Martin, F., Vilgalys, R., Bonito, G., 2017. Comparative genomics of *Mortierella elongata* and its bacterial endosymbiont *Mycoavidus cysteinexigens*. Environ. Microbiol. 19, 2964–2983. https://doi.org/10.1111/1462-2920.13669.

Vahdatzadeh, M., Deveau, A., Splivallo, R., 2015. The role of the microbiome of truffles in aroma formation: A meta-analysis approach. Appl. Environ. Microbiol. 81, 6946–6952. https://doi.org/10.1128/AEM.01098-15.

Vahdatzadeh, M., Splivallo, R., 2018. Improving truffle mycelium flavour through strain selection targeting volatiles of the Ehrlich pathway. Sci. Rep. 8. https://doi.org/10.1038/s41598-018-27620-w.

Ye, L., Li, Q., Fu, Y., Sourzat, P., Tan, H., Zou, J., Zhang, B., Li, X., 2018. Host species effects on bacterial communities associated with the fruiting bodies of *Tuber* species from the Sichuan Province in Southwest China. Mycol. Prog. 17, 833–840. https://doi.org/10.1007/s11557-018-1397-2.