Investigating the influence of truffle´s microbiome and genotype on the aroma of truffle fungi

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1 Summary

Truffles (*Tuber* spp.) are belowground forming fungi that develop in association with roots of various host trees and shrubs (i.e. hazel, oak and cistus). Their fruiting bodies are considered as prized delicacies and are renowned for their enticing aromas which vary considerably, even within truffles of the same species. This aroma variability might be attributed to factors such as geographical origin, degree of fruiting body maturation, truffle genotype (genetic background of the fruiting bodies) and microbiome (microbial communities that colonise truffle fruiting bodies) which often co-vary. Although the influence of specific factors is highlighted by several studies, discerning the contribution of each factor remains a challenge since it requires an appropriate experimental design as well as a representative sample size for statistical analysis. The primary purpose of this thesis was to gain insight into the influence of truffle's genotype and microbiome on truffle aroma.

This doctoral thesis is comprised of four chapters. Chapter1 (Vahdatzadeh et al., 2018) aimed to exclusively elucidate the influence of truffle genotype on truffle aroma by investigating the aroma of nine mycelial strains of the white truffle *Tuber borchii*. We also assessed whether strain selection could be employed to improve the human-perceived truffle aroma. Quantitative differences in aroma profiles among strains could be observed upon feeding of amino acids. Considerable aroma variabilities among strains were attributed to important truffle volatiles, many of which might be derived from amino acid catabolism through the Ehrlich pathway. Through this pathway, an amino acid undergoes deamination and forms an α-keto acid, followed by decarboxylation and formation of an aldehyde and subsequent reduction/oxidation to an alcohol/acid. ^{13}C-labelling experiments confirmed the existence of the Ehrlich pathway in truffles for leucine, isoleucine, methionine, and phenylalanine, which resulted in essential truffle odorants. Sensory analyses further demonstrated that the human nose perceived these aroma variabilities and can differentiate among strains. Our results illustrated the influence of truffle genotype on truffle aroma and showed how strain selection could be used to improve the human-perceived truffle aroma.

In chapter 2 the existing knowledge on the composition of bacterial community of four commercially relevant truffle species, *Tuber magnatum, Tuber melanosporum, Tuber aestivum and T. borchii*, was compiled using meta-analysis approach (Vahdatzadeh et al., 2015). We highlighted the endemic microbiome of truffle as well as similarities and differences in the composition of microbial community within species at various phases of their life cycle. Furthermore, the potential contribution of truffle microbiome in the formation of truffle odorants was studied. Our findings showed that truffle fruiting bodies harbour complex microbial community composed of bacteria, yeasts, filamentous fungi, and viruses with bacteria being the dominant group. Regardless of truffle species, the composition of endemic microbiome of fruiting bodies appeared very similar and was dominated by α-Proteobacteria class. However, striking differences were observed in the bacterial community composition at various stages of the life cycle of truffle. The community composition in the ectomycorrhizae strongly differed from the truffle fruiting bodies but was very close to the soil communities and mainly affiliated to the Actinobacteria class. Our analyses further suggested that odorants common to many truffle species might be produced by both truffle fungi and microbes, whereas specific truffle odorants might be derived from microbes only. Nevertheless, disentangling the origin of truffle odorants is very challenging, since acquiring microbe-free fruiting bodies are currently not possible. Furthermore, it requires the identification of aroma precursors of specific truffle odorants and the elucidation of their biosynthetic pathways in truffle fruiting bodies.

Chapter 3 (Splivallo et al., 2019) further characterises truffle-associated bacterial communities of a large number of fruiting bodies of the black truffle *T. aestivum* from two different orchards (Swiss and French) using high-throughput amplicon sequencing. It aimed at defining the native microbiome in this truffle species, evaluating the variability of their microbiome across orchards and assessing factors that shape assemblages of the bacterial communities (i.e. abiotic and biotic, such as truffle genotype, maturity, collection season, and spatial distance). The dominant bacterial communities in *T. aestivum* revealed to be similar in both orchards: although a large portion of fruiting bodies were dominated by the α-Proteobacteria class (*Bradyrhizobium* genus) similar to other so far-assessed truffle species, in few cases β-Proteobacteria (*Polaromonas* genus), or Sphingobacteria (*Pedobacter* genus) were

found to be predominant classes. Moreover, factors shaping bacterial communities influenced the two orchards differently, with spatial location within the orchard being the main driver in Swiss orchard and collection season in the French one. Surprisingly, in contrast to other fungi, truffle genotype and the degree of fruiting body maturity seemed not to contribute in shaping the assembly of truffle microbiome. Altogether, our data highlighted the existence of heterogeneous bacterial communities in *T. aestivum* fruiting bodies which are dominated by either of the three bacterial classes and mainly by the α-Proteobacteria class, irrespective of geographical origin. They further illustrated that determinants driving the assembly of various bacterial communities within truffle fruiting bodies are site-specific.

Truffles are highly perishable delicacies with a short shelf-life (1-2 weeks), and their aroma changes profoundly upon storage. Since truffle aroma might be at least partially produced by the truffle microbiome, chapter 4 (Vahdatzadeh et al., 2019) focuses on assessing the influence of the truffle microbiome on aroma deterioration of *T.aestivum* during post-harvest storage. Specifically, volatile profile and bacterial communities of fruiting bodies collected from four different regions (three in France and one in Switzerland) were studied over nine days of storage. Our findings demonstrated the gradual replacement of dominant bacterial classes in fresh truffles (α-Proteobacteria, β-Proteobacteria, and Sphingobacteria) by food spoilage bacteria (members of γ-Proteobacteria and Bacilli classes), regardless of the initial diversity of the bacterial classes. This shift in the bacterial community also correlated with changes in volatile profiles, and markers for truffle freshness (i.e. dimethyl sulfide, butan-2-one) and spoilage (i.e. 2- and 3-methylbutan-1-ol, and 2-phenylethan-1-ol) could be identified. Ultimately, network analysis illustrated possible links among those volatile markers and specific bacterial classes. Our data showed that storage deeply influenced the composition of bacterial community as well as aroma of truffle fruiting bodies. They also illustrated the correlation between the shift in truffle microbiome, from commensal to detrimental, and the change of aroma profile, possibly leading to the loss of fresh truffle aroma.

Overall, the work undertaken in this thesis demonstrated the presence of diverse truffle-associated bacterial communities which are mainly dominated by the α-Proteobacteria

class and to a lesser extent by β-Proteobacteria, or Sphingobacteria classes in fresh fruiting bodies (endemic truffle microbiome). It was also shown that various factors shaped the composition of fruiting bodies-associated bacterial communities differently depending on the local conditions. The results also highlighted the potential involvement of bacteria in truffle aroma formation and suggested that the loss of fresh truffle aroma upon storage might be due to the shift from commensal to detrimental bacteria. Lastly, the findings of this thesis revealed that truffle genotype and microbiome had a stronger influence on truffle aroma than previously believed. These results can be promising in two aspects of the truffle industry. Firstly, instead of spore infestation in nurseries, production of truffles with higher consumer acceptance might be achieved through the inoculation of seedlings with mycelial strains associated with more desired aromas. Secondly, truffle shelf life might be prolonged, and its aroma can be possibly maintained through the conservation of the native microbiome by applying targeted preservation techniques.

Nevertheless, further studies are required to advance our understanding of truffle fruiting body formation, and of how the complex bacterial communities are recruited from the surrounding soil. Moreover, other factors which might be involved in the shaping of the assemblages of bacterial community and the functions of these microbes in the biology of truffles and vice versa remain to be investigated.

2 Deutsche Zusammenfassung

Trüffel (*Tuber* spp.) sind unterirdische Pilze, die sich durch Symbiose mit den Wurzeln verschiedener Wirtsbäume und -sträucher (z.B. Haselnuss, Eiche und Zistrose) entwickeln. Ihre Fruchtkörper sind essbare Pilzorgane mit Sporen, die durch sexuelle Fortpflanzung entstehen. Tatsächlich sind Trüffel heterothallische Organismen, was bedeutet, dass sie zwei Paarungstypen (mütterlich und väterlich) für die Fortpflanzung benötigen. Das Paarungsereignis tritt ein, wenn der mütterliche Paarungstyp, der Pflanzenwurzeln in einem symbiotischen Organ besiedelt, das als Ektomykorrhizen bekannt ist, auf ein Individuum des entgegengesetzten (väterlichen) Paarungstyps trifft. Dieses Ereignis führt zur Bildung eines haploiden Fruchtkörpers, der Sporen beider Paarungsarten enthält. Molekular basierte Daten deuten auf die Existenz von etwa 180 Trüffelarten in verschiedenen Regionen der Welt hin, von denen etwa 30 kommerziell gehandelt werden. Die Preise reichen von einigen hundert Euro pro kg für die günstigsten Trüffelarten (z.B. der schwarze Sommer-Trüffel *Tuber aestivum* und der weiße Bianchetto-Trüffel *Tuber borchii*), bis hin zu Tausenden von Euro für die teuersten (z.B. der weiße Piemont-Trüffel *Tuber magnatum* und der schwarze Perigord-Trüffel *Tuber melanosporum*).

Trüffel-Fruchtkörper gelten als begehrte Delikatesse und sind bekannt für ihre einzigartigen Aromen, die teilweise aus Mikroben gewonnen werden. Tatsächlich beherbergen Trüffel-Fruchtkörper eine vielfältige mikrobielle Gemeinschaft aus Bakterien, Hefen, filamentösen Pilzen und Viren (Trüffel-Mikrobiom). Dieses sogenannte trüffelassoziierte Mikrobiom wird von Bakteriengemeinschaften dominiert, die das äußere und innere Gewebe des Fruchtkörpers stark besiedeln (sog. Peridium bzw. Gleba). Die Identität und Vielfalt dieser Mikroben ist jedoch noch wenig erforscht, da nur wenige Studien trüffelassoziierte Bakteriengemeinschaften bestimmt haben und nur eine geringe Anzahl von Fruchtkörpern berücksichtig wurde. Daher sind die Faktoren, die die Zusammensetzung des Trüffelmikrobioms antreiben, noch nicht geklärt.

Flüchtige Stoffe, die für die charakteristischen Gerüche von Trüffeln verantwortlich sind, sind eine Mischung aus Aldehyden, Ketonen, Aromaten und vor allem schwefelhaltigen

flüchtigen Bestandteilen. Einige dieser flüchtigen Stoffe scheinen nicht nur bei den meisten Trüffelarten, sondern auch bei anderen Pilzen und Pflanzen (z.B. 2- und 3-Methylbutanal, 2- und 3-Methyl-1-butanol) verbreitet zu sein, während andere ausschließlich von einer oder wenigen Trüffelarten emittiert werden (z.B. 2,4-Dithiapentan in *T. magnatum*). Die ausgeprägten Aromavariabilitäten wurden sowohl bei verschiedenen Trüffelarten, als auch bei Trüffeln einer Art beobachtet. Diese Aromavariabilität kann auf Faktoren wie geografische Herkunft, Reifegrad des Fruchtkörpers, Trüffelgenotyp (genetischer Hintergrund der Fruchtkörper) und Mikrobiom zurückgeführt werden, die oft kovariieren. Obwohl der Einfluss spezifischer Faktoren durch mehrere Studien hervorgehoben wird, bleibt die Beurteilung des Beitrags jedes Faktors eine Herausforderung, da er ein geeignetes experimentelles Design sowie einen repräsentativen Stichprobenumfang für die statistische Analyse erfordert. Das Hauptziel der vorliegenden Doktorarbeit war es, Einblicke in den Einfluss des Genotyps und des Mikrobioms der Trüffel auf das Aroma der Trüffel zu gewinnen.

Diese Doktorarbeit besteht aus vier Kapiteln. Kapitel 1 (Vahdatzadeh et al., 2018) zielte darauf ab, ausschließlich den Einfluss des Trüffelgenotyps auf das Trüffelaroma zu untersuchen, indem das Aroma von neun myzelialen Stämmen der weißen Trüffel *T. borchii* untersucht wurde. Zudem wurde untersucht, ob die Stammauswahl zur Verbesserung des vom Menschen wahrgenommenen Trüffelaromas eingesetzt werden kann. Bei der Zugabe von Aminosäuren zum Myzel konnten quantitative Unterschiede in den Aromaprofilen unter den Sorten beobachtet werden. Diesen erheblichen Aromavariabilitäten unter ~~konnte~~ den Stämmen wurden wichtigen ~~flüchtigen~~ Aromen wie 2- und 3-Methyl-1-butanol und ihren Aldehyden, Phenylacetaldehyd, Methional, Dimethylsulfid und Dimethyldisulfid zugeschrieben werden, von denen viele aus dem Aminosäurekatabolismus über die Aminosäuregärung (Ehrlich pathway) stammen könnten. Bei der Aminosäuregärung wird eine Aminosäure desaminiert und bildet eine α-Ketosäure, gefolgt von der Decarboxylierung und Bildung eines Aldehyds und der anschließenden Reduktion/Oxidation zu einem Alkohol/Säure. ^{13}C-Markierungsexperimente bestätigten die Existenz der Aminosäuregärung in Trüffeln für Leucin, Isoleucin, Methionin und Phenylalanin. Sensorische Analysen zeigten weiterhin, dass die menschliche Nase

diese Untersuchende im Aromaprofile wahrnehmen und zwischen den Sorten unterscheiden kann. Die Ergebnisse veranschaulichten den Einfluss des Trüffelgenotyps auf das Trüffelaroma und zeigten, wie die Sortenauswahl genutzt werden kann, um das vom Menschen wahrgenommene Trüffelaroma zu verbessern.

In Kapitel 2 wurden die vorhandenen Erkenntnisse über die Zusammensetzung der Bakteriengemeinschaft von vier kommerziell relevanten Trüffelarten, *T. magnatum, T. melanosporum, T. aestivum und T. borchii*, mit Hilfe eines Meta-Analyse-Ansatzes zusammengetragen (Vahdatzadeh et al., 2015). Es wurde das endemische Mikrobiom von Trüffeln sowie Ähnlichkeiten und Unterschiede in der Zusammensetzung der mikrobiellen Gemeinschaft innerhalb der Arten in verschiedenen Phasen des Trüffellebenszyklus hervorgehoben. Darüber hinaus wurde der mögliche Beitrag des Trüffelmikrobioms zur Bildung von Trüffelgeruchstoffen untersucht. Die Ergebnisse zeigten, dass unabhängig von der Trüffelart die Zusammensetzung des endemischen Mikrobioms der Fruchtkörper sehr ähnlich war und von der Klasse α-Proteobakterien dominiert wurde. Allerdings wurden in verschiedenen Phasen des Lebenszyklus von Trüffeln markante Unterschiede in der Zusammensetzung der Bakteriengemeinschaft beobachtet. Die Gemeinschaftszusammensetzung in der Ektomykorrhizae unterschied sich stark von den Trüffel-Fruchtkörpern, war aber sehr ähnlich wie die Bodengemeinschaften und hauptsächlich an die Actinobakterien-Klasse gebunden. Die Analysen deuteten weiter darauf hin, dass die für viele Trüffelarten üblichen Geruchsstoffe sowohl von Trüffelpilzen als auch von Mikroben produziert werden können, während spezifische Trüffelgeruchsstoffe nur von Mikroben abgeleitet werden können. Dennoch bleibt es eine große Herausforderung, die exakte Herkunft der Trüffelgeruchsstoffe zu identifizieren, da der Erwerb mikrobenfreier Fruchtkörper nicht möglich ist. Darüber hinaus erfordert dies die Identifizierung von Aromavorläufern spezifischer Trüffelgeruchsstoffe und die Aufklärung ihrer biosynthetischen Bahnen in Trüffelfruchtkörpern.

Kapitel 3 (Splivallo et al., 2019) charakterisiert weiterhin trüffelassoziierte Bakteriengemeinschaften einer großen Anzahl von Fruchtkörpern der schwarzen Trüffel *T. aestivum* von zwei verschiedenen Trüffelfarmen (Schweiz und Frankreich)

unter Verwendung von Amplikonsequenzierung mit hohem Durchsatz. Ziel war es, das native Mikrobiom in dieser Trüffelart zu identifizieren, die Variabilität ihres Mikrobioms innerhalb der Plantagen zu untersuchen und Faktoren zu bewerten, die die Ansammlungen der Bakteriengemeinschaften (d.h. abiotisch und biotisch, wie Trüffelgenotyp, Reife, Erntesaison und räumliche Entfernung) prägen. Die dominanten Bakteriengemeinschaften in *T. aestivum* erwiesen sich in beiden Plantagen als ähnlich: Obwohl ein großer Teil der Fruchtkörper von der Klasse α-Proteobakterien (Gattung *Bradyrhizobium*) dominiert wurde, ähnlich wie bei anderen bisher untersuchen Trüffelarten, wurden in wenigen Fällen β-Proteobakterien (Gattung *Polaromonas*) oder Sphingobakterien (Gattung *Pedobacter*) als dominante Klassen identifiziert. Darüber hinaus sind die Hauptfaktoren welche die Bakteriengemeinschaften prägen unterschiedlich: auf der schweizer Plantage war die räumliche Lage entscheidend, während auf der französischen Plantage der Erntezeitpunkt entscheidend war. Überraschenderweise schienen, im Gegensatz zu anderen Pilzen, der Trüffelgenotyp und der Grad der Fruchtkörperreife nicht zur Gestaltung des Trüffelmikrobioms beizutragen. Insgesamt zeigten die Daten die Existenz heterogener Bakteriengemeinschaften in *T. aestivum* Fruchtkörpern, die von einer der drei Bakterienklassen und vor allem von der Klasse α-Proteobakterien dominiert wurden, unabhängig von der geografischen Herkunft. Die Daten veranschaulichten weiterhin, dass die Determinanten, welche die Bildung verschiedener Bakteriengemeinschaften in Trüffel-Fruchtkörpern antreiben, standortspezifisch sind.

Trüffel sind leicht verderbliche Lebensmittel mit kurzer Haltbarkeit (1-2 Wochen), deren Aroma sich während der Lagerung stark verändert. Da das Trüffelaroma zumindest teilweise durch das Trüffelmikrobiom erzeugt werden kann, konzentriert sich Kapitel 4 (Vahdatzadeh et al., 2019) auf die Beurteilung des Einflusses des Trüffelmikrobioms auf das Aromaprofil von *T. aestivum* während der Lagerung. Insbesondere das Aromaprofil und die bakterielle Zusammensetzung der Fruchtkörper, die aus vier verschiedenen Regionen (drei in Frankreich und eine in der Schweiz) stammten, wurden während der Lagerung von neun Tagen untersucht. Die Ergebnisse zeigten den schrittweisen Austausch von dominanten Bakterienklassen in frischen Trüffeln (α-Proteobakterien, β-Proteobakterien und Sphingobakterien) mit lebensmittelverderbliche Bakterien (Mitglieder der Klassen γ-Proteobakterien und

Bacilli), unabhängig von der anfänglichen Vielfalt der Bakterienklassen. Diese Verschiebung in der Bakteriengemeinschaft korrelierte auch mit Veränderungen in den Aromaprofilen, und es konnten Marker für Trüffelfrische (z.B. Dimethylsulfid, Butan-2-on) und Verderb (z.B. 2- und 3-Methyl-1-butanol, und 2-Phenylethanol) identifiziert werden. Schließlich veranschaulichte eine Netzwerkanalyse mögliche Zusammenhänge zwischen diesen Markern und den spezifischen Bakterienklassen. Daten zeigten, dass die Lagerung die Zusammensetzung der Bakteriengemeinschaft sowie das Aroma der Fruchtkörper der Trüffel stark beeinflusst. Außerdem veranschaulichten die Daten den Zusammenhang zwischen der Verschiebung des Trüffelmikrobioms und der Änderung des Aromaprofils, welches möglicherweise zum Verlust des frischen Trüffelaromas führt.

Insgesamt zeigten die Ergebnisse dieser Arbeit das Vorhandensein von verschiedenen trüffelassoziierten Bakteriengemeinschaften, die hauptsächlich von der Klasse α-Proteobakterien und in geringerem Maße von β-Proteobakterien oder Sphingobakterien Klassen in frischen Fruchtkörpern (endemisches Trüffelmikrobiom) dominiert werden. Es zeigte sich auch, dass verschiedene Faktoren die Zusammensetzung der fruchtkörper-assoziierten Bakteriengemeinschaften je nach den lokalen Bedingungen unterschiedlich prägten. Zudem deutet die mögliche Beteiligung von Bakterien an der Bildung des Trüffelaromas darauf hin, dass der Verlust von frischem Trüffelaroma bei der Lagerung auf die Verschiebung des Trüffelmikrobioms zurückzuführen sein könnte. Zusammenfassend machen die Ergebnisse deutlich, dass sowohl Trüffel-Genotyp als auch Mikrobiom einen stärkeren Einfluss auf das Trüffelaroma haben als bisher angenommen. Die Ergebnisse der vorliegenden Doktorarbeit können in zwei Aspekten für die Trüffelindustrie vielversprechend sein. Erstens könnte eine höhere Verbraucherakzeptant erreicht werden, indem eine Kontrollierte Produktion von Trüffeln durchgeführt wird bei der Setzlinge mit Trüffelmyzel beimpft werden, sodass das gewünschte Aroma der resultierenden Trüffel kontrollierbar wird. Zweitens könnte die Haltbarkeit des Trüffels verlängert werden, wenn das native Mikrobiom durch die Anwendung der gezielter Konservierungstechniken aufrecht erhalten werden kann.

Dennoch sind weitere Studien erforderlich, um unser Verständnis der Trüffelfruchtkörperbildung und der Rekrutierung der komplexen Bakteriengemeinschaften aus dem umgebenden Boden zu verbessern. Darüber hinaus müssen noch andere Faktoren untersucht werden, welche die Gesamtheit der Bakteriengemeinschaft beeinflussen und an der Funktion dieser Mikroben in der Biologie der Trüffel beteiligt sein könnten.

3 Introduction

3.1 What are truffles?

Truffles are edible ascomycetes fungi, forming hypogeous fruiting bodies in a symbiotic relationship with various host trees, mainly hazel, oaks, pines (Harley and Smith, 1983) as well as some shrubs including cistus (Fontana and Giovanetti, 1978). As a result of the symbiosis, truffle establishes the ectomycorrhizae network on the surface of the root tip of the host plant and a Hartig latticework among the root's cortical cells which allow the exchange of growth-limiting nutrients among them. Truffles provide the host with nitrogen, and phosphorous and receive carbohydrates in exchange (Le Tacon et al., 2013; Bonito and Smith, 2016). Also, in rare instances, truffles have been observed as non-ectomycorrhizae forming within orchid roots (i.e. with a member of *Neottieae, Epipactis microphylla*) (Selosse et al., 2004).

Truffle-forming fungi exist not solely within the Ascomycota, but also among other phyla (i.e. Basidiomycota). They have developed in at least 13 individual orders within a distinct taxonomical lineage (Fig. 1). Historically, truffle-forming fungi belonging to the Basidiomycota phylum were called "false truffles" to distinguish them from truffles within the Ascomycota. Among the latter phylum, only hypogeous fruiting bodies which belong to the order Pezizales, the family *Tuberaceae*, and the genus *Tuber* are considered as the "true truffles" (Jeandroz et al., 2008; Bonito and Smith, 2016).

Besides the hypogeous fruiting bodies, the order Pezizales includes epigeous species with a symbiotic or saprotrophic lifestyle (Mello et al., 2006). In fact, the family *Tuberaceae* evolved from the epigeous saprotrophic ancestors in the last 140 million years ago (Bonito et al., 2013; Murat et al., 2018b). Factors driving the evolution have remained obscure, but it is hypothesised to be due to an adaptation to animal grazing and/or environmental stress (drought, frost, and fire) (Thiers, 1984; Bruns et al., 1989). Interestingly, main evolutionary transitions in truffle-forming species occur in the ectomycorrhizae forming fungi lineage (Trappe et al., 2009) which suggests that symbiosis with the host plant has been the main driver in the diversity of truffle evolution.

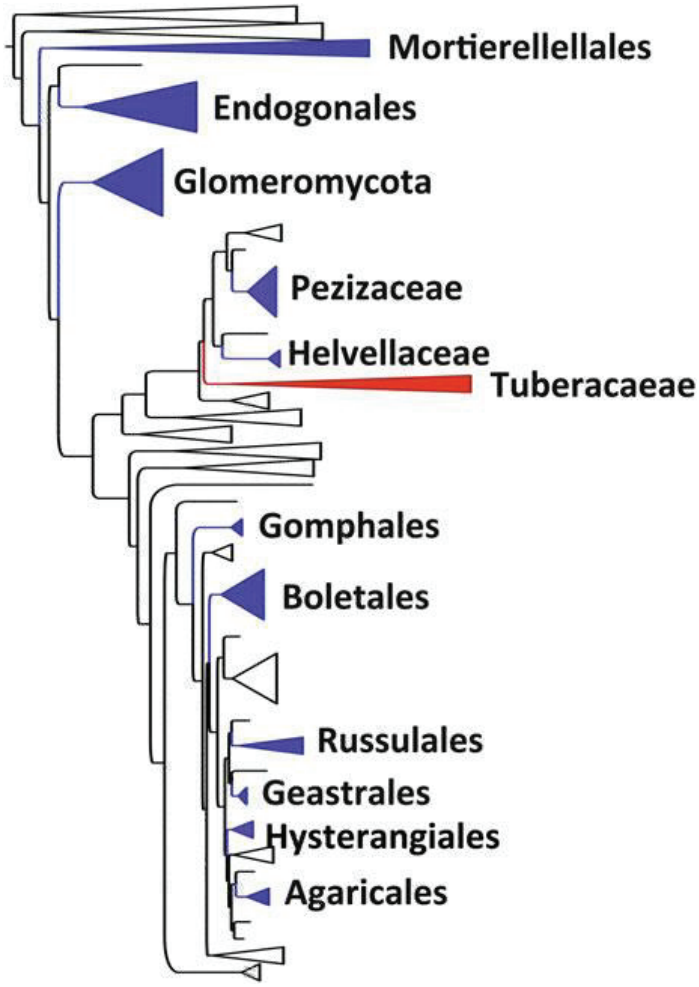

Figure 1. Phylogenetic tree of main orders of truffle-forming fungi. Fungal orders and families, in case of the order Pezizales, that form truffle fruiting bodies are shown. Orders and the family *Tuberacaeae* including the "true truffles" are colour-coded in blue and red, respectively (adapted from Bonito &Smith., 2016).

Despite high genetic diversity, *Tuber* species demonstrate important similar genomic features (Murat et al., 2018b). A recent study comparing the genome of several truffle species to saprotrophic Pezizyomycetes (i.e. *Morchella importuna* and *Ascobolus immersus*) revealed *Tuber*-specific genomic features including 1) decrease of genes encoding lignocellulose (main constituents of the plant's cell wall) degrading enzymes, 2) high content of transposable regions, and 3) high abundance of genes related to volatiles metabolism (Murat et al., 2018b). These specific features indicate the limited ability of truffle to degrade the host-plant cell wall (due to the evolution from saprotrophic

to symbiotic lifestyle), an accelerated gene turnover and a pungent aroma released from the truffle fruiting bodies.

In fact, for centuries, truffle fruiting bodies have been mainly praised in the culinary world because of their unique aromas and regarded as a food delicacy (Splivallo and Culleré, 2016). From more than 180 *Tuber* species existing worldwide, about 30 are commercialised (Bonito et al., 2010). They are classified as a luxury commodity due to excessively high pricing, ranging from a few hundred up to thousands of Euros per kilogram. Many commercially relevant truffle species are endemic to Europe including the "Périgord black truffle" (*Tuber melanosproum* Vittad.), the "summer truffle" (*Tuber aestivum* Vittad.), and its morphotype the "Burgundy truffle" (*Tuber uncinatum*) (Weden et al., 2005), the "white Piedmont truffles" (*Tuber magnatum* Pico), and the "bianchetto truffle" (*Tuber borchii* Vittad.) (Hall et al., 2007). Other local European species such as *Tuber brumale* Vittad., *Tuber macrosporum* Vittad. and *Tuber mesentericum* Vittad. exist but have a small market (Merényi et al., 2016; Benucci et al., 2016). Nevertheless, some of these truffle species (i.e. *T. melanosporum*) have been introduced in several other countries such as USA, Australia, New Zealand, and Chile (Bonito et al., 2010; Hall and Haslam, 2012; Jeandroz et al., 2008; Lefevre, 2012).

In the USA, there are some endemic truffle species of economic value (i.e. *Tuber lyonii* Butters, *Tuber gibbosum* Harkn. and *Tuber oregonense* Trappe, Bonito, and Rawl) (Bonito et al., 2011, 2013). Several truffle species (i.e. *Tuber indicum, Tuber sinense, Tuber himalayense, Tuber formosanum)* are harvested in China (Cooke and Massee, 1982; Zhang and Minter, 1988; Tao et al., 1989; Hu, 1992) of which some appear to have similar morphological characteristics as the valuable species but have inferior gastronomical value and aroma complexity such as the Chinese black truffle *T. indicum* and *T. melanosporum* (Riousset et al., 2001; Culleré et al., 2013a).

Besides the above-described species, there are also desert truffles which belong to the *Terfeziaceae* family (and not the *Tuberaceae*). Desert truffles, as the name suggests, are native to the arid and semi-arid areas (i.e. North Africa, Eastern Mediterranean, and the Middle East) where they are appreciated for their flavour and aroma (Díez et al., 2002). In comparison to the "true truffles", they lack the pungent aroma and have a lower market value (Loizides et al., 2012).

However, truffles are not only a gourmet mushroom but also a versatile model organism to study genomics, population genetics, ectomycorrhizae symbiosis, and the evolution history of *Tuber* species considering several sequenced *Tuber* genomes especially in the recent years (i.e. *T. magnatum*, *T. melanosporum*, *T. aestivum*, and *T. borchii*) (Martin et al., 2010; Rubini et al., 2011b; Payen et al., 2014; Murat et al., 2018a, 2018b).

3.2 Truffles: life cycle, distribution, and cultivation

Truffles life cycle

The fungi belonging to Ascomycota, the largest fungal phylum, produce an enormous number of spores through sexual and asexual reproduction. They inherited their name through the development of their sexual spores (ascospores) in a sac-like structure known as a “ascus” (plural asci). Yet, not all the ascomycete’s fungi have sexual reproduction and are unable to form asci. Asexual reproduction, as the dominant form of dissemination within Ascomycota, is mainly through conidia formation or in some cases budding (i.e. yeasts).

Truffle has an intriguing life cycle which so far has not been fully understood due to several challenges. Replicating the full-lifecycle of truffles in vitro is difficult. The formation of fruiting bodies (ascocarps) requires 5 – 20 years, which in itself is a complex belowground process involving interactions with appropriate host trees and a vast array of soil microorganisms (Rubini et al., 2014; Le Tacon et al., 2016; Selosse et al., 2017).

Several studies have attempted to gain insight into the mechanism involved in the sexual life of *Tuber* species. *T. melanosporum* was mainly used as a model system to study truffle biology and evolution which resulted in breakthroughs including 1) the first sequenced genome of *Tuber* species (Martin et al., 2010), 2) discovery of two mating type genes (maternal and paternal), this is particularly relevant since for a long time truffles were believed to be homothallic (i.e. presence of both mating genes in a single strain) (Riccioni et al., 2008; Rubini et al., 2011b), and 3) disclosure of the progressive exclusion of one mating type (paternal) by the opposite mating type (maternal) on the colonised roots which has been proposed to be linked to the vegetative incompatibility (Rubini et al., 2011b, 2014; Selosse et al., 2017).

Truffles fruiting bodies, as heterothallic species, require two mating types for sexual reproduction (Fig. 2). The maternal individual (haploid mycelium with either MAT 1-1 or MAT 1-2 from germinated ascospores) colonises the short roots of the tree and establishes the ectomycorrhizae (symbiotic organ). The paternal individual (haploid mycelium of the opposite mating type) has an unknown ecological niche (Rubini et al., 2011b). It has been suggested that a haploid mycelium produced from germinated ascospores or mitotic conidia, plays a role as the paternal individual (Rubini et al., 2011b; Healy et al., 2013), similar to numerous other Ascomycetes (Nelson, 1996).

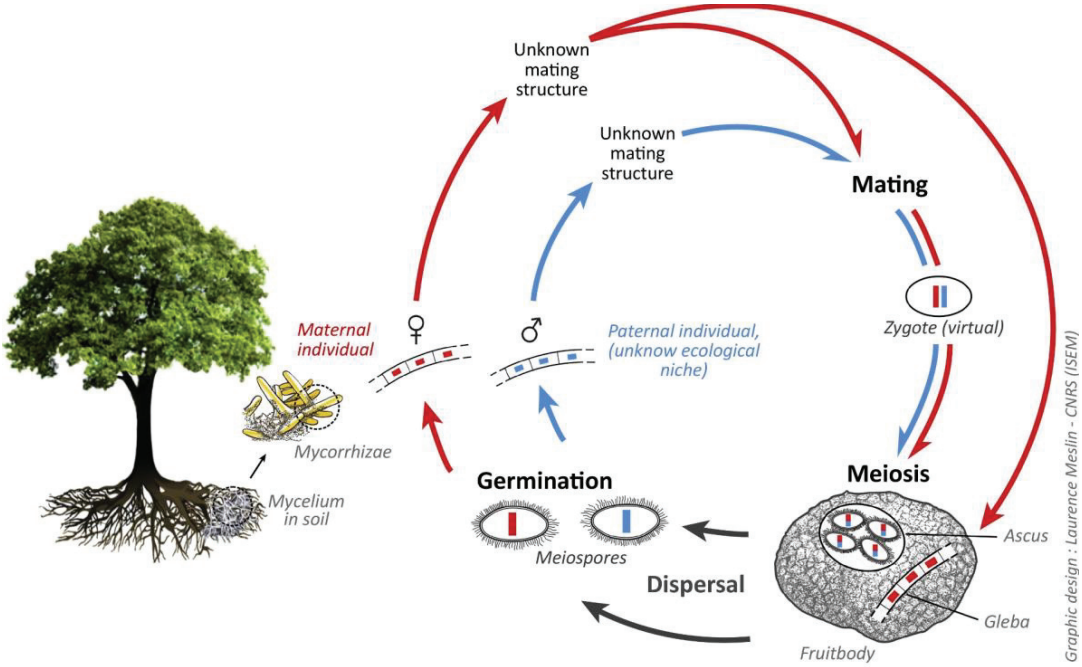

Figure 2. Sexual reproduction of Périgord black truffle *T. melanosporum*. Maternal and paternal mating types are colour-coded in red and blue, respectively. The sequestrate fruiting body (the gleba), the edible fungal-organ, originate from the haploid maternal individual (with either MAT 1-1 or MAT 1-2 allele). The gleba host ascospores (meiospores) in compartments so-called asci (each ascus contains four spores) resulted from meiosis division of virtual zygote made of the mating event between haploid individuals of opposite mating types (MAT 1-1 and MAT 1-2) (adapted from Selosse et al., 2017).

Mating occurs when these two individuals meet. This event leads to the formation of diploid virtual zygotes which consequently undergo meiosis and produce haploid

ascospores (also called meiospores) bearing both mating types. The maternal individual protects the spores by supplying niche (inner part of the fruiting body, so-called the gleba) and nutrition (via ectomycorrhizae formation), whereas the paternal individual contributes exclusively with the genetic materials of ascospores (Rubini et al., 2014; Belfiori et al., 2016; Selosse et al., 2017).

The factors stimulating fruiting body formation, mating structure, underlying exclusion of one mating type, and ecological niche of paternal mating types remain to be discovered. A better understanding of sexual reproduction of *Tuber* species paves the way for the development and management of natural and artificial truffle plantations which will be subsequently discussed.

Distribution of truffles

Despite the wide geographical distribution, “true truffles” are naturally found almost only in the northern hemisphere (Jeandroz et al., 2008). Truffles are endemic to all Europe (Le Tacon, 2016). Some species can be found in North America, several regions in Asia (China, and India) (Bonito et al., 2013), Africa (Morocco) (Ceruti et al., 2003), and the Middle East (Iran). Several factors might have shaped the current natural distribution of *Tuber* spices in the northern hemisphere, such as climate conditions, soil properties, and co-migration with their host plants (Murat et al., 2004).

The most valuable truffle species have various natural habitats. The black truffle *T. melanosporum* is endemic to Europe and can be mainly found in France, Italy, and Spain (Ceruti et al., 2003). However, as previously mentioned, it has been introduced to many other countries (Bonito et al., 2010; Hall and Haslam, 2012; Jeandroz et al., 2008; Lefevre, 2012). Also, *T. magnatum* has a narrow natural distribution, confined in the south-east of France to Italy and central European countries (i.e. Serbia, Slovenia, Croatia, and Hungry) (Ceruti et al., 2003; Hall et al., 2007). Both of these species seem to be very susceptible to the summer drought and winter frost (Le Tacon, 2016). *T. borchii* is broadly distributed in Europe. Similarly, *T. aestivum* is present throughout Europe, North Africa (i.e. Morocco) (Ceruti et al., 2003), and Iran. Contrary to *T. magnatum*, *T. aestivum* offers a wide range of ecological adaptability (i.e. soil properties, climate condition, and host trees) which led to the wide distribution of this species (Le Tacon, 2016).

Truffles cultivation

The first commercial cultivation of fungi dates back to the 17th century when botanist Joseph Pitton de Tournefort began the production of edible basidiomycete saprotrophic species *Agaricus bisporus* in France (Flegg et al., 1985). However, cultivation of mycorrhizal-forming fungi is relatively new since controlling the sexual reproduction (necessary for the fruiting body formation) is hardly possible.

The limited natural distribution of truffles and the increase in worldwide demand for truffles on the food market, together with a decrease of productivity have encouraged scientist to broaden the research about truffles and their cultivation (Mello et al., 2006). As a result of these efforts, considerable progress has been made to unravel the truffle life cycle, as well as substantial improvement truffle cultivation methods over the last 40 years (Rubini et al., 2007; Martin et al., 2010; Rubini et al., 2011a, 2011b; Murat, 2015; Selosse et al., 2017).

In 1808, the earliest cultivation method of Périgord black truffle was developed by Josef Talon in France via infesting acorns by sowing them in the rooting zone of oak trees which were already producing truffles and relocating the acorns afterwards (mother plant technique) (Hall et al., 2007). Cultivations of *Tuber* species were initiated by Bruno Fassi, Anna Fontana and Mario Palenzona in Italy in the late 1960s, after discovering the symbiotic nature of truffle. They found the relation between several *Tuber* species with their host trees (Fontana, 1967; Fassi and Fontana, 1967, 1969; Palenzona, 1969; Fontana and Palenzona, 1969; Fontana and Fasolo Bonfante, 1971). They induced the synthesis of *Tube*r ectomycorrhizae via the inoculation of *Pinus strobus* with *T. maculatum* (Fontana, 1967; Fassi and Fontana, 1967, 1969); *Corylus avelana* with *T. aestivum*, *T. brumale*, and *T. melanosporum* (Palenzona, 1969); and *Phyllostachys nigra* with *T. brumale* (Fontana and Fasolo Bonfante, 1971). Moreover, they succeeded to produce *T. maculatum* fruiting bodies in association with *P. strobus* in the culture pot (Fassi and Fontana, 1969). Unfortunately, the latter result could not be reproduced for *T. maculatum* and any other *Tuber* species (Splivallo, 2008). Modern truffle cultivation started in 1972 when Gérard Chevalier and Jean Grente developed and scaled up the inoculation technique of seedlings (Murat, 2015). Inoculation of seedlings was performed via three methods: mother plant technique, mycelial and spore inoculation.

Although the mycelial inoculation showed some potential (Chevalier, 1973), spore inoculation was the chosen technique for commercially produced seedlings (Zambonelli et al., 2015). In 1973, the first inoculated seedlings with *Tuber* species were commercially available in France (Murat, 2015). Nowadays, inoculated seedlings are produced by many nurseries, mainly in Europe and to a lesser extent in non-European countries such as New Zealand, USA, Australia, and Chile using techniques developed in Italy and France (Chevalier and Grente, 1979; Hall and Haslam, 2012).

The most successfully cultivated truffle worldwide is *T. melanosporum*, followed by *T. aestivum* and *T. borchii* (Murat, 2015; Zambonelli et al., 2015). However, cultivation of *T. magnatum* is more complex and was recently started by nurseries in Italy and France. The complexity lies in identifying *T. magnatum* mycorrhizae, which increased the risk of contamination with undesirable truffle species (i.e. *T. maculatum* and *T. borchii*) until the recent developments of modern molecular DNA identification tools. Also, *T. magnatum* forms a low mycorrhization degree of host trees in the nursery, which appears to fade away after plantation (Mello et al., 2001; Rubini et al., 2001; Murat et al., 2005; Hall et al., 2007).

In general, truffle fruiting bodies are currently harvested in both naturally established truffle fields as well as artificial plantations. The latter has a major contribution to the truffle market of France, Italy, and Spain. For instance, in France, over 80% of Périgord black truffle collected are from artificial plantations (Murat, 2015).

3.3 Diversity and ecology of truffle volatiles

Truffle volatiles diversity

To date, more than 300 volatiles from eleven truffle species have been reported including sulfur-containing volatiles, aldehydes, ketones, and aromatic compounds (Mauriello et al., 2004; Zeppa et al., 2004; Splivallo et al., 2007a; Culleré et al., 2010; Splivallo et al., 2011). Some of these volatiles appear to be common not only to most truffle species but also to other fungi and plants (i.e. 2- and 3-methylbutanal, 2- and 3-methylbutanol, and 1-octen-3-ol) while others are emitted exclusively by one or few species like 2,4-dithiapentane in *T. magnatum* and 1-methoxy-3-methylbenzene limited

to black truffle species (Fiecchi et al., 1967; Díaz et al., 2003; Mauriello et al., 2004; March et al., 2006; Splivallo et al., 2011; Splivallo and Ebeler, 2015).

Biosynthesis of truffle volatiles

The origin of most truffle odorants remains elusive and a matter of speculation. They might be produced either by the mycelium itself (i.e. from amino acids and/or fatty acids catabolism), by the associated microbes colonising the truffle fruiting body (so-called microbiome) or by both (mixed origin) (Splivallo et al., 2011; Splivallo and Ebeler, 2015). However, genome sequence-derived data of *Tuber* species (i.e. *T. melanosporum*, *T. aestivum*, and T*. magnatum*) suggests that truffles possess all the genes required for the biosynthesis of their key volatiles (Martin et al., 2010; Murat et al., 2018b). The major groups of truffle odorants and their potential biosynthetic routes will be discussed subsequently.

Sulfur-containing volatiles might be derived from L-methionine

Sulfur-containing volatiles (hereafter sulfur volatiles) are the key contributor to truffle aroma, as they emit the typical sulfurous note perceived from various *Tuber* species and possess a very low sensory detection threshold (Guadagni et al., 1963). Some sulfur metabolites commonly produced by many *Tuber* species include, dimethyl sulfide, dimethyl disulfide, dimethyl trisulfide while others are species-specific such as 3-methyl-4,5-dihydrothiophene and 2-methyl-4,5-dihydrothiophene, characteristic of *T. borchii* (Bellesia et al., 2001; Culleré et al., 2010; Splivallo et al., 2011; Liu et al., 2013; Splivallo et al., 2015; Splivallo and Ebeler, 2015).

Formation of sulfur volatiles in truffle, similar to the budding yeast *Saccharomyces cerevisiae* or other microorganisms producing sulfur volatiles in the food industry (i.e. ascomycetous yeasts and bacteria species), might be based on the methionine catabolism via two hypothetical “standard” and “non-standard” Ehrlich pathways. In the “standard” Ehrlich pathway an amino acid undergoes deamination and forms an α-keto acid, followed by decarboxylation and formation of an aldehyde and subsequent reduction/oxidation to an alcohol/acid (Hazelwood et al., 2008).

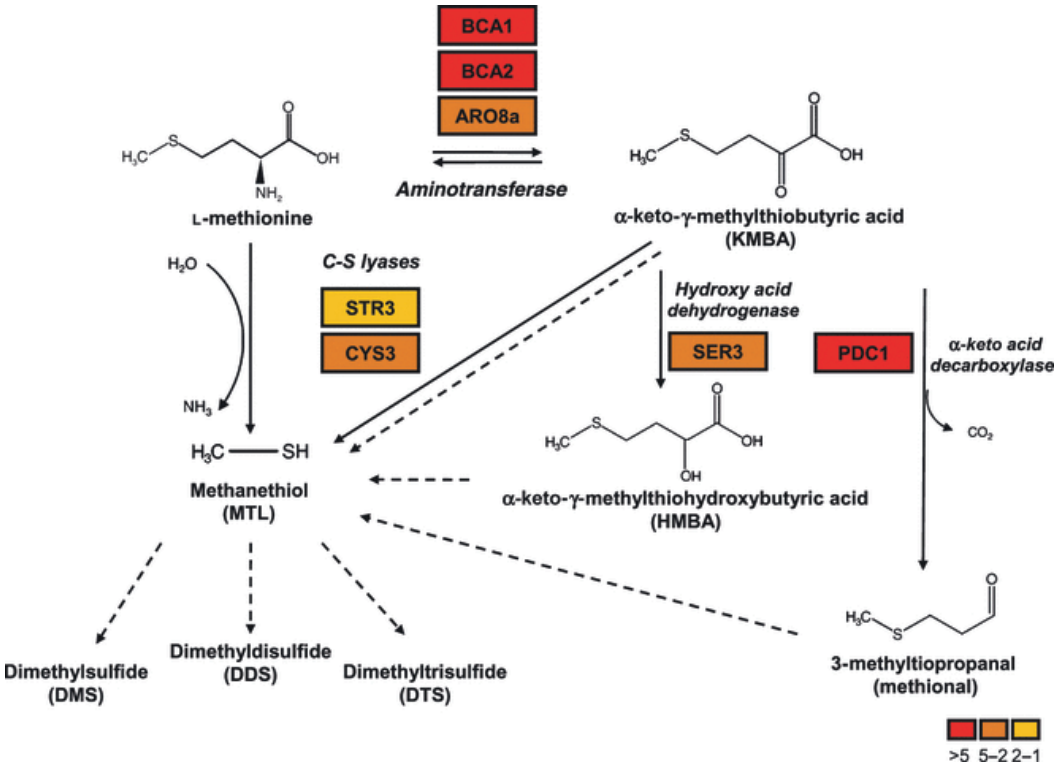

Figure 3. Hypothetical biosynthetic pathways and genes for the most important sulfur volatiles in *Tuber melanosporum*. The scheme illustrates the catabolism of methionine via "standard" and "non-standard" Ehrlich pathway. Solid and dashed arrows demonstrate enzyme-mediated and spontaneous reactions, respectively. Candidate genes are colour-coded to reflect the fruiting-body (FB) expression levels with respect to free-living mycelia (FLM) (data based on the Martin et al., 2010), with a colour gradient ranging from red (FB/FLM > 5) to yellow (2 ≤ FB/FLM ≥ 1); as shown at the bottom (adapted from Splivallo et al., 2011).

Through this pathway (Fig.3), methionine is first deaminated into the 4-methylthio-2-oxobutyric acid (KMBA) supported by branched-chain and aromatic aminotransferase enzymes (i.e. TmelBCA1, TmelBCA2, and TmelARO8a). Then it is followed by either decarboxylation into 3-methylsulfanylpropanal (methional) or reduction into 4-methylthio-2-hydroxybutyric acid (HMBA) (by TmelPDC1, an α-keto acid decarboxylase or TmelSER3, a hydroxy acid dehydrogenase enzymes, respectively), with a final conversion into methanethiol (MTL) via chemical decomposition reactions. The formation of MTL might also occur through non-enzymatic or enzymatic reactions from KMBA (Arfi et al., 2006; Liu et al., 2008). Candidate enzymes potentially involved in this reactions include C-S lyases such as cystathionine β and γ-lyases which also

contribute to sulfur metabolisms in plant and bacteria (i.e. production of flavour compounds by lactic acid bacteria in fermented dairy products) (Jones et al., 2003; Liu et al., 2008). In the “non-standard” Ehrlich pathway, methionine might directly be degraded into MTL by candidate enzymes such as cystathionine or methionine γ-lyases (Arfi et al., 2006). The latter enzymes convert the sulfur-containing amino acids to several metabolites, including sulfur volatiles in several bacteria and plants such as *Arabidopsis thaliana* (Sato and Nozaki, 2009). However, it has not been found in truffles or other fungi (Sato and Nozaki, 2009). MTL resulting from either “standard” or “non-standard” pathways can spontaneously decompose to dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide.

Except for the methionine γ-lyases, genes encoding all the candidate enzymes involved in sulfur volatiles biosynthesis have been proposed in the genome of *Tuber* species (Martin et al., 2010; Murat et al., 2018a). Interestingly, these genes are sustained and overexpressed in the fruiting body compared with free-living mycelium. Nevertheless, the biosynthetic pathway of more complex sulfur volatiles such as 2,4-dithiapentane (*T. magnatum*) and thiophene derivatives (*T.borchii*) and their precursor(s) are yet to be investigated.

Branched-chain and aromatic volatiles synthesis from non-sulfur amino acids catabolism

Another important group of truffle odorants are branched-chain and aromatic volatiles including, 2- and 3-methylbutanal, and 2-phenylacetaldehyde. It has been suggested that these truffle odorants, similar to Saccharomyces yeasts, might be synthesised from the catabolism of free amino acids (i.e. leucine, isoleucine, and phenylalanine) through the Ehrlich pathway. Although the truffle mycelium supplemented with amino acids showed to produce many of these volatiles, confirmation of this pathway in truffle is yet to be proven through the feeding of labelled amino acids (Splivallo and Maier, 2012; Liu et al., 2013; Xiao et al., 2015). Nevertheless, possible candidate genes contributing to the Ehrlich pathway have been suggested following the genome sequencing of *Tuber* species (Martin et al., 2010; Murat et al., 2018a).

Truffle fruiting bodies contain several free amino acids. In the Périgord black truffle, the level of free amino acid changed during the maturation. For instance, the concentration of alanine and isoleucine significantly increase, whereas cysteine decreased by more than a half. This might explain the emergence or disappearance of specific volatiles during maturation (Harki et al., 2006).

Volatiles synthesis from fatty acids

Eight-carbon-containing volatiles (hereafter C_8 volatiles) represent an important group of truffle odorants, as they are key constituents of fungal aroma and typical mushroom flavour (Combet et al., 2006). The most common C_8 volatile is 1-octen-3-ol which exists as two stereoisomers of R and S. Fungi emit mainly the R enantiomer which imparts “typical fungal” odour, in comparison to the S form which has “mouldy, grassy” smell (Combet et al., 2006). In mushrooms, 1-octen-3-ol is synthesised from linoleic acid (Wurzenberger and Grosch, 1984), while arachidonic acid serves as a precursor in some plants (*Marchantia polymorph* and moss *Physcomitrella patens*) (Senger et al., 2005; Kihara et al., 2014). This suggests that truffle fungi produce the R form of this compound from linoleic acid. Interestingly, linoleic acid appears to be by far the major fatty acid constituent in *T. melanosproum*, irrespective of the degree of maturation (Harki 2006). In ascomycetous fungi, *Aspergillus nidulans*, (Eidam) G. Winter, linoleic acid is converted to intermediate compound 10-hydroperoxyoctadecadienoic acid (10-HPOD) via the dioxygenases enzyme Ppo, which might be transformed nonenzymatically to 1-octen-3-ol (Garscha and Oliw, 2009; Brodhun et al., 2010).
The sequenced genome of the black truffle proposed the existence of two copies of Ppo enzymes (Martin et al., 2010). However, elucidating this pathway in truffles require feeding labelled precursors and fully identifying the supporting enzymes.

Ecological functions of truffle volatiles

Truffles interact with other organisms at different phases of the life cycle, and these include plants, mammals, and insects via volatiles known as signal molecules (Menotta et al., 2004).

Truffles-plant interactions

Primary communication of truffle might initially occur between truffle mycelia and the host plant before the ectomycorrhizae formation (during the vegetative phase) (Miozzi et al., 2005) and continue during the mycorrhizal and the reproductive phases (fruiting body formation). Although numerous volatiles have been found in truffles at various life stages (Tirillini et al., 2000; Menotta et al., 2004; Mauriello et al., 2004; Zeppa et al., 2004; Splivallo et al., 2007b; Culleré et al., 2010), the function of few of them in truffle-plant interaction has been described and is mostly based on speculation. For instance, it is suggested that C_8 volatiles such as 1-octen-3-ol, emitted by truffles, inhibit plant development by stimulating changes in the root morphology and bleaching of seedlings (Menotta et al., 2004; Splivallo et al., 2007b). Similarly, morphological changes in the root of *Arabidopsis thaliana* (i.e. thickening the roots tip, and elongation of the root hair) has been described due to hormonal signals ethylene and indole-3-acetic acid emitted by truffle mycelium (Splivallo et al., 2007b). Such signalling molecules might be involved in facilitating the association between truffle-host plant and ectomycorrhizae formation. They could also be involved in suppressing the plants' growth around the symbiont and development of a burnt zone (so-called brûlé) observed in some truffle species such as *T. melanosporum* (Napoli et al., 2010).

Truffles-mammal interactions

Interaction of truffles with mammals is essential for truffle spores dissemination. While other ascomycetous fungi use forcible spore discharge for their dispersal (Trail, 2007), truffle disperse their spores through the faeces of mycophagist animals of which small mammals (i.e. wild pig, squirrels, and rodents) are of main importance (Talou et al., 1990; Maser et al., 2008; Trappe and Claridge, 2010). Truffle fruiting bodies attract mammals through the production of intense aromas. Therefore, trained dogs and less often pigs which possess a refined olfactory system are used by truffle hunter to locate truffles belowground. Talou et al. (1990) have shown that pigs and dogs are attracted by dimethyl sulfide (Talou et al., 1990). As dogs are capable of discerning truffle species, other attractants besides dimethyl sulfide should be involved, but they are yet to be found.

Truffles-insect interactions

Besides mammals, two insects, a fly (*Suillia pallida*) and a beetle (*Leiodes cinnamomea Panzer*), are also able to localise truffles (Hochberg et al., 2003; Maser et al., 2008). Interestingly, the fly is also used by truffle hunters. It has been proposed that similar to mammals; truffle flies might be attracted by dimethyl sulfide (Pacioni et al., 1991).
Truffle beetle feeds on several truffles to complete its life cycle (Arzone, 1970; Newton, 1984) but truffle volatiles that attract *L. cinnamomea* have not been identified. Hochberg et al. (2003) demonstrated that the adult beetles are not attracted to the ripe fruiting body under laboratory conditions. Therefore, volatiles emitted by unripe truffles might act as attractants (Hochberg et al., 2003). Whether these insects are only fungivores or they are also involved in the truffle spore dissemination is unknown.

Factors influencing truffle aroma

Truffle aroma qualitatively and quantitatively varies not only among the different truffle species (Mauriello et al., 2004) but also within truffles of the same species. Differences in the aroma profile might be associated with series of biotic and abiotic factors including degree of fruiting body maturity (Zeppa et al., 2004), genotype (Splivallo et al., 2012; Molinier et al., 2015), stages of truffle's life cycle (mycelium vs. fruiting body) (Splivallo et al., 2007a), truffle microbial community (Splivallo et al., 2015; Splivallo and Ebeler, 2015), post-harvest storage (Bellesia et al., 1998; Falasconi et al., 2005; Aprea et al., 2007; Pennazza et al., 2013; Culleré et al., 2013b; Splivallo et al., 2015), and geographical origin (Gioacchini et al., 2005; Vita et al., 2015).
The difficulty arises when studying the effect of individual determinants as they often co-vary. For instance, aroma variability was observed among T*. magnatum* fruiting bodies harvested from different regions of Italy (Gioacchini et al., 2005; Vita et al., 2015). However, this variability might be attributed not only to the distinct geographical origins but also to other drivers such as microbiome, maturity, soil characteristics, season and importantly genotype.
Genotypic variabilities within fruiting bodies occur among the distinct regions as well as within the same orchard in different seasons. Since truffle has a high genetic turn over (Murat et al., 2018b), the occurrence of fruiting bodies of the same genotype (clone) in

consecutive years seldom happens (Murat et al., 2013; Molinier et al., 2015). In *T. aestivum*, profound differences in the concentration of C_4 (four carbon-containing volatiles) and C_8 volatiles (i.e. 1-octen-3-ol and 2-butanone) between the fruiting bodies harvested few centimetres from each other has been attributed to the genetic variabilities (Splivallo et al., 2012; Molinier et al., 2015).

Among other factors that could influence the aroma variability, maturity has been reported to affect the aroma composition of *T. borchii*. In fresh truffles, thiophene volatiles proposed to occur only in fully mature fruiting bodies (Zeppa et al., 2004). Also, these compounds were demonstrated to be synthesised exclusively by bacteria colonising the fruiting bodies and their concentration correlated with the bacterial abundance upon storage at room temperature (Splivallo et al., 2015). The evolution of the structure of the bacterial community during maturation in *T. melanosporum* (Antony-Babu et al., 2014) illustrates the link between maturation and bacterial community. Hence, determining the contribution of maturity and bacterial community is only possible by investigating both factors simultaneously in fresh and stored truffles.

Truffles are perishable commodity with a short shelf-life (one to two weeks). Post-harvest storage conditions (i.e. time, temperature) profoundly affect truffle aroma as highlighted by several studies (Bellesia et al., 1998; Falasconi et al., 2005; Aprea et al., 2007; Pennazza et al., 2013; Culleré et al., 2013b; Splivallo et al., 2015). This is, for instance, illustrated for dimethyl sulfide concentration, which decreased upon cold storage for two months in *T. melanosporum* fruiting bodies (Culleré et al., 2013b). Since storage also influence the truffle microbial community (Saltarelli et al., 2008; Rivera et al., 2010b) possible linkage among these drivers remains to be discovered. This is one of the goals of this thesis, which will be addressed in chapter 4.

Another factor which also affects the truffle aroma is the stage of the life cycle. Truffles emit various volatiles during different developmental stages. For example, thiophene derivatives are exclusively produced by *T. borchii* throughout the reproductive phase (fruiting bodies) and not during the vegetative phase (mycelium) (Splivallo et al., 2015). Generally, a higher number of volatiles are detected from truffle fruiting bodies compared to the axenic cultures of truffle mycelium. This might be due to the higher number of activated metabolic pathways in fruiting bodies or absence of complex

interacting microbiome in axenic mycelial culture (Gabella et al., 2005; Splivallo et al., 2007a).

Challenges to study aroma variability in truffles

Taken together, several drivers might influence the aroma variability in truffles, which often are interconnected. The study of the contribution of each factor remains a challenge since it requires an appropriate experimental design as well as a representative sample size for statistical analysis. The latter, considering the harvest yield fluctuations and rarity of clones would be very complicated.

Although nowadays is possible to reproduce the vegetative (axenic mycelial culture) and symbiotic phases (inoculated seedling) of several truffle species under nursery or controlled laboratory conditions, the reproductive phase cannot be triggered in the laboratory or artificial orchard. This makes it more difficult to tune the yield and to grow truffles with defined genetic material or microbial community. Study of the potential influence of factors, namely truffle genotype and microbiome on truffle aroma, is the main objective of this thesis.

3.4 Truffles as food

Mushrooms are part of the human diet since the early stage of civilisation and very popular in many cuisines because of their distinctive sensory properties, nutritional values and possible health benefits (Cuppett et al., 1998; Mattila et al., 2002; Ferreira et al., 2010). Indeed, nowadays, several tons of the most familiar type of mushroom, *A. bisporus*, is produced in mushroom farms worldwide (Royse et al., 2017).

Truffles, except for their distinctive aroma, have a similar chemical composition to other fungi (Holtz and Schisler, 1972; Brennan et al., 1975; Bokhary and Parvez, 1993). Chemical characteristics of truffles including, nutritional profile and human-sensed truffle aroma, will be subsequently discussed.

Nutritional profile of truffles

Truffles are a rich source of proteins, minerals, fibres, sulfur-containing amino acids, and fatty acids but they are poor in lipids, and their water content is relatively low

compared to other edible fungi (Bokhary and Parvez, 1993; Harki et al., 2006; Saltarelli et al., 2008; Wang and Marcone, 2011; Cherno et al., 2013). The nutritional content of truffles varies among species. For instance, a study for chemical profiling of four truffle species, *T. melanosporum*, *T. aestivum*, *T. magnatum*, and *T. borchii,* showed that dry matter (DM) consists of high protein content ranged from 8.7 to 24.0%, with *T. melanosporum* and, *T. magnatum* containing the lowest and highest protein concentration, respectively (Saltarelli et al., 2008). The same study showed low carbohydrate content compared to protein for all species (2% to 6% of DM), with *T. aestivum* possessing the highest carbohydrate content (Saltarelli et al., 2008). Nevertheless, a biochemical study of *T. melanosproum* demonstrated that carbohydrate content is significantly affected by the degree of maturity and its concentration strongly increased (i.e. about three folds) during maturation. This can be explained by the abundance of melanised spores rich in carbohydrate in mature truffle (Harki et al., 2006). In comparison to truffles, the most common mushroom species *A. bisporus* has a mild crude protein content (19% of DM), whereas carbohydrates content are dominant and contribute up to 56.5% of DM (Cherno et al., 2013). Carbohydrate in truffles, similar to other fungi species, is mainly composed of soluble polysaccharide with glucose being the major constituent (Harki et al., 2006; Cherno et al., 2013; Kruzselyi and Vetter, 2014). High crude fiber content seems to be a characteristic of truffles, which is higher than those reported in asco- and basidiomycetous fungi (Kalač, 2009; Kruzselyi and Vetter, 2014). Analysis of free amino acids revealed that truffles contain all essential as well as sulfur-containing amino acids (i.e. methionine, and cysteine) (Harki et al., 2006). The mineral spectrum of truffles is similar to the majority of edible mushrooms including potassium, phosphorus, calcium, and magnesium, which together constitute 97.85% of the minerals in *T. aestivum*. They also contain some important microelements, such as iron (Harki et al., 2006; Saltarelli et al., 2008; Kalač, 2009; Kruzselyi and Vetter, 2014). Lipids in truffles are predominantly composed of fatty acid; linoleic acid being the major component in truffle whereas for *A. bisporus*, oleic acid is dominant (Harki et al., 2006; Cherno et al., 2013). The concentration of linoleic acid showed to increase three folds during maturation (Harki et al., 2006).

Overall, truffles possess low energy level (1224 KJ/100 g DM) similar to other edible fungi (Mattila et al., 2002). They are a rich source of many elements, such as protein, minerals and are free of toxic components.

Human-sensed truffle aroma

Volatiles imparting characteristic truffle aroma are blends of hydrocarbons composed of diverse functional groups and sulfur compounds (Ney and Freitag, 1980; Claus et al., 1981). Yet, human-sensed truffle aroma, like other foods, is due to a small portion of these volatiles, so-called aroma active compounds or odorants (Culleré et al., 2010; Liu et al., 2012; Dunkel et al., 2014; Splivallo and Ebeler, 2015). For instance, volatile profile of a single truffle species is composed of typically 30 to 60 volatiles of which 15 to 20 are aroma active compounds (Bellesia et al., 1998; Díaz et al., 2003; Mauriello et al., 2004; Gioacchini et al., 2005; March et al., 2006; Splivallo et al., 2007a; Culleré et al., 2010; Schmidberger and Schieberle, 2017).

In recent years, the number of reported volatiles of various truffle species is growing as extraction techniques advance and become more sensitive. The extraction of volatiles is mostly carried out using headspace techniques such as solid-phase microextraction (HS-SPME) and purge and trap (Talou et al., 1987; Bellesia et al., 1998; Díaz et al., 2003; Mauriello et al., 2004; Zeppa et al., 2004; Falasconi et al., 2005; Gioacchini et al., 2005; March et al., 2006). Although gas chromatography-mass spectrometry (GC-MS) provides useful information about volatiles, it does not determine whether these volatiles are odorants. The odorant composition can be identified through a sensory technique, called GC-olfactometry (GC-O), which uses the human nose as a detector (van Ruth, 2001).

Odorants of some commercially relevant species (*T. magnatum, T. melanosprum, T. aestivum and T. borchii*) have been evaluated using GC-O method (Jansen et al., 2003; Piloni et al., 2005; Culleré et al., 2010). Piloni et al. (2005) used this method to determine the impact of the volatiles formerly detected by GC-MS on the aroma of white truffle *T. magnatum*. The total of five sulfur odorants were identified of which dimethyl sulfide (truffle (Piloni et al., 2005)), along with bis(methylthio)methane (garlic-like (Schmidberger and Schieberle, 2017)) showed to be important aroma contributors of this species (Piloni et al., 2005). Also, another study reported for the first time 1-

pyrroline (amine-like, sperm-like (Schmidberger and Schieberle, 2017).) as a key odorant of *T. magnatum* and *T. aestivum* based on the odour activity values (OAVs, ratio of the concentration of a compound to its odour thresholds) (Schmidberger and Schieberle, 2017). Culleré et al. (2010) compared the aroma profile of the black truffles, *T. melanosporum* and *T. aestivum* and concluded that the aroma composition of *T. melanosporum* is more complex and intense than *T. aestivum*. Although these species showed some similarity in their key odorants (i.e. dimethyl sulfide, dimethyl disulfide, 3-methyl-1-butanol), higher content of 2,3-butanedione, ethyl butyrate in *T. melanosporum* and methional in *T. aestivum* was observed (Culleré et al., 2010). A similar study compared the aroma composition of the Chinese black truffle *T. indicum* to its morphological analogue but of higher gastronomical value, *T. melanosporum*, and reported much higher aroma intensity of the latter species. In contrast to *T. melanosporum*, the major aroma contributor to the overall aroma of *T. indicum* includes mainly C_8 volatiles (1-octen-3-one, and 1-octen-3-ol), esters, and in a lower amount sulfur volatiles (dimethyl sulfide and dimethyl disulfide) (Culleré et al., 2013a). Higher content of C_8 volatiles in *T. indicum* might be a useful tool to differentiate among these species and to avoid the fraud. Different sensory techniques (i.e. aroma extract dilution analysis (AEDA) and omission test) applied to *T. indicum* as well as two other Chinese species *T. himalayense*, and *T. sinense* confirmed 1-octen-3-ol (mushroom-like (Combet et al. 2006)) as a major contributor to the Asian truffle species (Liu et al., 2012). An olfactometric study of white truffle *T. borchii* demonstrated that sulfur compounds of microbial origin, thiophene derivatives (mainly 3-methyl-4,5-dihydrothiophene) are the major aroma contributor to the aroma of this species (Splivallo et al., 2015; Splivallo and Ebeler, 2015).

Overall, these studies suggest that sulfur volatiles are among the most important aroma contributors to the human-sensed truffle aroma.

3.5 Truffle-associated microbiome: diversity and potential roles

Diversity of truffle microbiome

Besides plants, mammals and insects, truffle interacts with microbes. Indeed truffles harbour complex microbial community composed of bacteria, yeast, filamentous fungi, and viruses at various stages of their life cycle (Barbieri et al., 2005, 2007; Buzzini et al., 2005; Pacioni et al., 2007; Stielow and Menzel, 2010; Stielow et al., 2011a, 2011b, 2012; Gryndler et al., 2013; Antony-Babu et al., 2014; Splivallo et al., 2015). Truffle-associated microbiome are dominated by bacterial community which heavily colonise the fruiting body peridium (outer surface) and gleba with densities of up to billions of bacterial cells per gram of fruiting bodies (Sbrana et al., 2002; Barbieri et al., 2005, 2007; Nazzaro et al., 2007; Saltarelli et al., 2008; Rivera et al., 2010a). Bacterial communities of a few commercially relevant species including *T. magnatum* (Barbieri et al., 2007), *T. melanosporum* (Antony-Babu et al., 2014), *T. borchii* (Barbieri et al., 2005; Splivallo et al., 2015) have been characterised using previously culture-dependent and nowadays mainly culture-free techniques are used (i.e. high-throughput sequencing). The bacterial community composition of gleba highlighted the existence of endemic truffle microbiome commonly dominated by bacteria belonging to the α-Proteobacteria class, *Bradyrhizobium* genus along with, to a lesser extent, members of other bacterial classes such as β and γ-Proteobacteria, Sphingobacteria, Bacteroidetes (Barbieri et al., 2005, 2007, 2016; Antony-Babu et al., 2014; Splivallo et al., 2015; Benucci and Bonito, 2016). The bacterial community of peridium, however substantially differed from the gleba but was similar to the soil community. It is suggested that truffles selectively attract and recruit their microbiome from the communities of surrounding soil since the early stage of fruiting body formation (Antony-Babu et al., 2014).

Factors driving the composition of truffle microbiome

Despite the similarities in the microbiome composition of truffles, major variabilities observed not only among various species but also within the same truffle species (Benucci and Bonito, 2016). These variations might be attributed to a series of intrinsic and extrinsic factors such as season, soil properties, fruiting body maturity, and genotype. In fact, it has been hypothesised that the variation in bacterial composition

of truffle might be linked to the degree of fruiting body maturity for *T. indicum*, *T. melanosporum*, and *T. borchii* (Citterio et al., 2001; Antony-Babu et al., 2014; Splivallo et al., 2015; Ye et al., 2018).

In addition to maturation, the stage of the truffle's life cycle has also been suggested to influence the composition of bacterial communities in *T. melanosporum* (Antony-Babu et al., 2014). The microbial composition of ectomycorrhizae was markedly distinct from the fruiting body but was very similar to the bulk soil and was dominated by Actinobacteria. Yet, the latter bacterial class is scarce in the fruiting body of black truffle (Antony-Babu et al., 2014). These differences might be associated with the diverged functions and biochemical composition of these organisms.

Another factor which possibly influences the composition of the bacterial community associated with the truffle fruiting body is the post-harvest condition. The latter has been investigated for a few species and only using culture-dependent methods (Saltarelli et al., 2008; Rivera et al., 2010b). For example, Rivera et al. (2010b) reported a shift in the community composition of *T. aestivum* and an increase of members belonging to the prevalent food spoilage bacteria, *Entrobacteriaceae* family, after 21 days of post-harvest storage at 4°C (Rivera et al., 2010b). Similarly, rapid growth in the population of cultivable bacteria (ca. 10^2 CFU/g) was also documented for fresh *Tuber* fruiting bodies namely *T. borchii*, *T. melanosporum* and *T. aestivum* conserved at 4°C only after 4-8 days (Saltarelli et al., 2008).

However, this picture of bacterial community composition is incomplete, since it represents only changes in the cultivable fraction of the bacterial community. To gain a comprehensive insight into the influence of these factors on the overall microbial community, implementing high throughput techniques to include uncultivated members is required. This is specifically one of the aims of this thesis (chapters 3 and 4).

Potential roles of truffle-associated microbiome

Despite the vast diversity of the truffle-associated microbiome, their functions and effect on truffle biology remained elusive and are poorly investigated. Several hypothetical roles for the truffle microbiome in development, maturity and aroma formation of various *Tuber* species (i.e. *T. magnatum*, *T. melanosporum*, and *T. borchii*) have been proposed

(Barbieri et al., 2000; Citterio et al., 2001; Sbrana et al., 2002; Splivallo et al., 2007b; Barbieri et al., 2010; Antony-Babu et al., 2014; Splivallo et al., 2015). The involvement of *T. borchii* associated bacteria in the formation of thiophene derivatives by biotransformation of unknown non-volatile precursor(s) has been demonstrated (Splivallo et al., 2015; Splivallo and Ebeler, 2015). Also, it has been proposed that bacterial family of *Bradyrhizobiaceae* associated with *T. magnatum* might contribute to fruiting body N_2 fixation (Barbieri et al., 2010). Other potential roles including improvement of truffle nutrition, ascocarp degradation and interaction with the other soil fungi have been attributed to strains of Actinobacteria isolated from *T. magnatum* fruiting body (Pavić et al., 2013).

Overall, truffle offers habitat to a diverse microbial community with mostly unknown functions, especially possible involvement in the production of the human-perceived truffle aroma which remains to be elucidated. This thesis aimed to investigate the latter in chapters 2 and 4.

3.6 Structure and aims of the doctoral thesis

Unique aromas of truffles are made of hydrocarbons with various functional groups and of sulfur atoms that vary considerably depending on the species. The aroma also varies within the same truffle species, and this variability might be linked to factors such as the geographical origin, maturation, and genetics of fruiting bodies (genotype) or the microbes colonising truffle fruiting bodies (microbiomes).

The studies carried out in this thesis were based on four working hypotheses: 1) The genetic background of truffles influences more than just a handful of volatiles as reported earlier in literature, 2) Microbiomes of truffle fruiting bodies might be responsible for the formation of numerous volatiles that are relevant for the human-sensed truffle aroma, 3) The composition of microbial communities colonising truffles might be influenced by truffle genotype, maturation and also orchard conditions, and 4) Truffle microbiomes might be involved in aroma deterioration upon post-harvest storage.

This thesis is structured in four chapters according to these working hypotheses. Chapter 1 (Vahdatzadeh and Splivallo, 2018) aimed at investigating the influence of truffle genotype on truffle aroma and the human aroma perception. This objective was addressed by quantifying the extent of aroma variability among axenic cultures of nine mycelial strains of the white truffle *T. borchii* and identifying the main volatiles causing these variabilities and their biosynthetic pathway (amino acids catabolism via the Ehrlich pathway). We also confirmed the existence of the Ehrlich pathway in truffles using ^{13}C labelled-amino acids. Moreover, we employed sensory analyses to investigate whether the human nose is capable of distinguishing the aroma variability among strains. The goals of chapter 2 (Vahdatzadeh et al., 2015) are twofold. First, using a meta-analysis approach, the data on the microbial community composition of economically relevant truffle species (i.e. *T. magnatum*, *T. melanosporum*, *T. borchii*, and *T. aestivum*) was compiled to illustrate similarities and differences in the composition of microbial community within species at various phases of their life cycle. Second, the possible involvement of truffle microbiome in the production of truffle odorants was assessed. Chapter 3 (Splivallo et al., 2019) focuses on the evaluating

the composition of bacterial communities of a large number of *T. aestivum* fruiting bodies collected over several years from two distinct orchards (in Switzerland and France) via high throughput amplicon sequencing. It also illustrates the variability among bacterial community structure across orchards and factors (i.e. intrinsic and extrinsic such as truffle genotype, fruiting body maturity, collection season, and spatial distance) driving the assembly of truffle-associated bacterial communities. The main aim of chapter 4 (Vahdatzadeh et al., 2019) is to investigate the potential contribution of truffle microbiome in truffle aroma deterioration upon post-harvest storage of *T. aestivum*. It demonstrates the influence of post-harvest storage on both volatile profile and bacterial community composition of *T. aestivum*. It also highlights the existence of freshness and spoilage volatile markers and correlates these markers to special bacterial groups. Finally, in the general discussion, the main findings of all studies are discussed, the remaining open questions raised and the scene for the future investigations set.

4 Discussion

4.1 Truffle genotype predominantly influences truffle aroma and the human aroma perception

Genetics has a high impact on sensory characteristics of various food products (Aharoni et al., 2004; Kreutzmann et al., 2008). Therefore, strain selection is widely used in the food industry to create specific aroma and flavour or to reduce off-flavour formation (H.E. Ayad et al., 2000; Tanous et al., 2002; Smit et al., 2005; Vilela-Moura et al., 2008). In this thesis (chapter 1), we showed the important effect of truffle genotype on aroma variability and how strain selection could be employed to enhance the human-sensed aroma of truffle mycelium.

Truffle aroma variability exists even among the same truffle species which might be associated with drivers such as degree of fruiting body maturity, geographical origin, truffle genotype, and microbiome (Zeppa et al., 2004; Gioacchini et al., 2005; Splivallo et al., 2012; Molinier et al., 2015; Vahdatzadeh et al., 2015). The study of the contribution of a single factor influencing the truffle aroma is a challenging task since they often co-vary. We removed the co-variance by studying the influence of genotype on the aroma of axenic cultures of nine mycelial strains of the white truffle species *T. borchii*.

Although the aroma profile of axenic mycelial strains of various *Tuber* species have been previously highlighted in several studies, none of them investigated aroma variability among the same mycelial strains (Tirillini et al., 2000; Splivallo et al., 2007b; Li et al., 2012; Liu et al., 2013; Du et al., 2014; Xiao et al., 2015). Our findings demonstrated significant quantitative differences in emitted volatiles among nine mycelial strains of *T. borchii* upon amino acids supplementation (i.e. methionine, leucine, isoleucine, and phenylalanine). A major part of this aroma variability appeared to be due to volatiles potentially derived from amino acid catabolism (Ehrlich pathway) namely 2- and 3-methyl butanal and their alcohols, 2-phenylacetaldehyde, benzaldehyde, methional, dimethyl disulfide and dimethyl trisulfide. However, a study previously suggested the contribution of genetic on truffle aroma variability for C_4 and

C_8 volatiles (i.e. 2-butanol and 1-octen-3-ol) in fruiting bodies of *T. aestivum* (Splivallo et al., 2012; Molinier et al., 2015). The discrepancy of these two studies might be attributed to the study system meaning differences between truffle fruiting bodies and axenic mycelial culture. Indeed, axenic truffle mycelium lack interacting microbes (i.e. bacteria and yeasts which are extensively explained in chapter 2) colonising truffle fruiting bodies which, besides truffle fungi, can produce the Ehrlich-derived volatiles (Vahdatzadeh et al., 2015). It has been reported that isolated yeasts from truffle fruiting body can produce volatiles from the Ehrlich pathway including 2- and 3-methylbutanol, dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide (Buzzini et al., 2005).

The existence of the Ehrlich pathway in truffles revealed by ^{13}C labelling

Amino acids catabolism through the Ehrlich pathway in budding yeast includes three steps of deamination resulting into an a-keto acid, decarboxylation and formation of an aldehyde followed by oxidation or reduction and production of alcohol or acid, respectively (Hazelwood et al., 2008). Several studies reported production of many Ehrlich-derived volatiles via inoculation of axenic mycelium of truffle by amino acids (Splivallo and Maier, 2012; Liu et al., 2013; Xiao et al., 2015). However, it has never been proven. We confirmed the existence of this pathway in *Tuber borchii* using ^{13}C-labeled amino acids for leucine, isoleucine, methionine and phenylalanine (Vahdatzadeh and Splivallo, 2018).

Methionine through two hypothetical pathways, “standard” and “non-standard”, leads to the formation of sulfur volatiles which involved genes have been proposed based on the sequenced genome of *Tuber* species (Martin et al., 2010; Splivallo et al., 2011; Murat et al., 2018b). The “standard” Ehrlich pathway (Fig.3) is initiated by deamination of methionine into the 4-methylthio-2-oxobutyric acid (KMBA), followed by either decarboxylation into 3-methylsulfanylpropanal (methional) or reduction into 4-methylthio-2-hydroxybutyric acid (HMBA) with the eventual production of methanethiol (MTL) via chemical reactions. The latter might be directly formed from KMBA via both chemical decomposition and non-chemical reactions. In the “non-standard” pathway, methionine might be converted directly to MTL via enzyme-supported reaction. In each of these pathways, there might be the ultimate formation of sulfur volatiles, namely dimethyl sulfide, dimethyl disulfide and dimethyl trisulfide through the chemical

decomposition of MLT (Splivallo et al., 2011). Our results support the existence of, at least, the “standard” pathway since methional was emitted from the axenic mycelium supplemented with ^{13}C labelled methionine. However, dimethyl sulfide, the most common sulfur volatile in truffle fruiting bodies, could not be detected from the axenic mycelium culture, which could be explained by either of the following two hypotheses. Firstly, our cultural condition did not favour the production of dimethyl sulfide, since different culture conditions reported to induce this compound in *T. melanosporum* and *T. formosanum* (Li et al., 2012; Du et al., 2014). Indeed, cultural conditions have been illustrated to affect the emission of volatiles significantly, to the extent that it outweighed the truffle species (Tang et al., 2009). Secondly, dimethyl sulfide might be only produced by the fruiting body microbiome (i.e. by several bacterial classes and also yeasts) (Buzzini et al., 2005; Vahdatzadeh et al., 2015, 2019) and not by the truffle mycelium similar to what has been reported for thiophene derivatives (Splivallo et al., 2015). Nevertheless, these speculations require further investigation.

The Ehrlich-derived volatiles influence the human aroma perception

The Ehrlich-derived volatiles are important food odorants meaning that they can be perceived by the human nose (Dunkel et al., 2014). For instance, dimethyl sulfide imparts truffle and sulfurous note (Culleré et al., 2010) or 2-phenylethan-1-ol confer rose-like smell (Splivallo and Culleré, 2016). However, the contribution of odorants to the human-sensed aroma depends on their concentration, which should be above their detection threshold (OAV>1) and also food matrix. Sulfur volatiles are the influential group of truffle odorants which possess a very low odour threshold (Guadagni et al., 1963) in comparison to the other odorants. In this regard, we confirmed the predominant contribution of sulfur volatiles in the human-sensed truffle aroma since panellists could distinguish between un-supplemented and methionine-supplemented samples and described it as sulfur/ garlicky (Vahdatzadeh and Splivallo, 2018). This result corroborates earlier findings demonstrating the key contribution of sulfur volatile in the human-perceived aroma of *T. borchii* fruiting body (Splivallo et al., 2015).

We further demonstrated that truffle genotype, besides being an important driver in the emission of volatiles, strongly affect the human aroma perception since panellists were

able to differentiate among the strains supplemented with the same amino acids (Vahdatzadeh and Splivallo, 2018). Studies on the influence of strains on the human aroma perception revealed comparable results for other foods (Romano et al., 2003; Leroy and De Vuyst, 2004; Aharoni et al., 2004; Marullo et al., 2006). The strains of the yeast culture, for instance, has been reported to have a major effect on the overall aroma characteristic and consequently, customer acceptability of wine (Romano et al., 2003).

Overall, our findings highlight the predominant influence of truffle genotype on truffle aroma variability and the human aroma perception. This might open not only a possibility to use mycelial strains with more desirable flavours for inoculation of seedlings in nurseries but also potentially providing the market with a higher quality of natural truffle flavour to replace synthetic flavour.

4.2 Truffle-associated microbiome might contribute to fresh truffle aroma and aroma deterioration upon storage

Endemic truffle microbiome diversity and potential functions

Microbes are widespread in ecosystems and interact with various hosts such as plants, animals, fungi and humans and play essential roles in their host ecology and lifecycle. Truffles harbour numerous but poorly understood microorganisms including bacteria, yeast, filamentous fungi and viruses (Barbieri et al., 2005, 2007; Buzzini et al., 2005; Pacioni et al., 2007; Stielow and Menzel, 2010; Stielow et al., 2011a, 2011b; Gryndler et al., 2013; Vahdatzadeh et al., 2015; Benucci and Bonito, 2016).

Bacteria are the dominant group of truffle-associated microbiome which colonise both outer and inner tissue of truffle fruiting body (peridium and gleba, respectively) with diverse microbial assemblages which is very divergent from each other (Antony-Babu et al., 2014; Vahdatzadeh et al., 2015). As highlighted in chapter 2, several studies showed that existence of the endemic microbiome in fresh fruiting bodies (gleba) of several *Tuber* species (i.e. *T. magnatum*, *T. melanosporum*, *T. borchii*) which are dominated by the α-Proteobacteria class mainly affiliated to the *Bradyrhizobium* genus (Antony-Babu et al., 2014; Vahdatzadeh et al., 2015; Benucci and Bonito, 2016; Ye et

al., 2018). In this thesis (chapter 3) by characterisation of bacterial communities composition of a large number of the black truffle *T. aestivum* collected over several years from two distinct orchards in Swiss and France, we confirmed the dominance of the α-Proteobacteria (*Bradyrhizobium* genus) also in this truffle species which corroborates previous results from various truffle species (Antony-Babu et al., 2014; Benucci and Bonito, 2016; Vahdatzadeh et al., 2015; Ye et al., 2018). Yet, in some cases (about 10 - 20 %), *T. aestivum* fruiting bodies illustrated a different pattern and were dominated by bacterial classes belonging to the β-Proteobacteria (*Polaromonas* genus), or Sphingobacteria (*Pedobacter* genus) (Splivallo et al., 2019; Vahdatzadeh et al., 2019). Interestingly fruiting bodies dominated by these genera showed a quite low richness of the communities (10 - 20 OTUs), indicating the complete invasion of the gleba by these genera and replacement of the *Bradyrhizobium* (Splivallo et al., 2019). Such variations in the microbiome have not been reported from other *Tuber* species which might be explained by a small number of assessed fruiting bodies.

Potential functions of truffle microbiome

Recurrent dominance of these microbial communities raises questions concerning their proven function in the truffle life cycle and ecology, which remains to be investigated. Some hypothetical roles have been proposed for several bacterial classes. It has been reported that isolated bacteria of the *Bradyrhizobium* genus from *T. magnatum* expressed the nitrogenase gene nifH and showed activity similar to the young legume nodules (Barbieri et al., 2010). This group of bacteria has been suggested to contribute to truffle nutrition during the reproductive phase (formation of fruiting body) (Barbieri et al., 2010). In spite of the presence of homologs of this gene in the microbiome of *T. melanosporum* (Antony-Babu et al., 2014), N_2 fixation activity was detected neither in immature nor mature fruiting body so far (Le Tacon et al., 2016). Other suggested functions from isolated bacteria from fruiting body includes, for instance, growth stimulation of *Tuber* mycelium, and inhibition of pathogenic fungi (Citterio et al., 2001; Sbrana et al., 2002; Dominguez et al., 2012; Gryndler et al., 2013, 2015; Antony-Babu et al., 2014; Deveau et al., 2016). Also, microbes might be involved in the elaboration of some truffle aroma compounds (Vahdatzadeh et al., 2015, 2019) as it has been

proven in a single case of sulfur volatiles characteristic of *T. borchii* (Splivallo et al., 2015). The latter potential role will be extensively discussed afterwards due to their potential importance in the human-sensed truffle aroma.

In general, our results highlighted that *T. aestivum* fruiting bodies mainly dominated by bacteria belonging to the a-Proteobacteria class and to a lower extent by members of the β-Proteobacteria and Sphingobacteria classes, irrespective of the geographical origin. Deployment of similar extensive sampling for other *Tuber* species to investigate whether the similar microbial pattern appears or whether this is specific to *T. aestivum* shall be the focus of further studies. Nevertheless, primary findings on *T. melanosporum* also propose a similar pattern (Deveau et al. unpublished). Furthermore, elucidation of the ecological functions of these microbes in truffle biology might lead to improvement of the truffle yield, preservation techniques and natural truffle flavour biotechnology.

Factors driving the structure of truffle bacterial communities

The assembly of bacterial communities associated with humans, plants, animals, and fungi might be driven by several factors such as host identity, genetics, and environmental factors as highlighted by several studies (Benson et al., 2010; Hacquard et al., 2015; Glynou et al., 2016; Pent et al., 2018; Matthews et al., 2019). In truffle fungi, factors shaping the structure of bacterial communities are poorly investigated (Pent et al., 2018).

Bonito 2016 compared the microbial community composition of four truffle species belonging to the genera *Tuber*, *Terfezia*, *Leucangium*, and *Kalapuya*, and reported the ubiquitous presence of a *Bradyrhizobium* OTU specifically associated with *Tuber* species. These authors concluded that truffle species strongly influence the composition of bacterial communities (Benucci and Bonito, 2016). However, variation in community composition exists not only among different species but also among truffles of the same species (Barbieri et al., 2016; Splivallo et al., 2019; Vahdatzadeh et al., 2019). This variation in the microbial community might be attributed to various factors. It might be artefactual, due to the applied methodologies to study microbial structure, for instance, culture-dependent vs. culture-free techniques such as high throughput sequencing (Sbrana et al., 2002; Barbieri et al., 2010; Deveau et al., 2016).

Another part of that variation might be partially due to natural variation in the composition of bacterial communities among truffle fruiting bodies of the same species. Nevertheless, a comprehensive study of the main determinants of truffle microbiomes and the extent to which they influence the bacterial community is missing.

In chapter 3 of this thesis, we carried out an extensive study to assess the relative importance of the main potential intrinsic and extrinsic factors (i.e. maturity, truffle genotype, mating type, collection season, and spatial location in the truffle orchard) driving the truffle-associated microbiome of *T. aestivum* collected from two distinct orchards (i.e. Swiss and France) over several years. Among other factors, host genotype has been reported as an important driver of bacterial community structure in several hosts (Benson et al., 2010; Pent et al., 2018). Yet, it was not the instance for *T. aestivum*, in which truffle genotype seemed not to contribute significantly to shaping bacterial community structure (Splivallo et al., 2019). This apparent contradiction suggests that the relative importance of each driver is host-specific. Also, truffle maturity might be an influential driver as the biochemical composition of fruiting body changes during maturation (Harki et al., 2006). In fact, the degree of maturity of truffle fruiting bodies has been suggested as a potential determinant of the bacterial composition of *T. borchii*, *T. indicum*, and *T. melanosporum* (Citterio et al., 2001; Antony-Babu et al., 2014; Splivallo et al., 2015; Ye et al., 2018). However, in *T. magnatum,* the structure of bacterial communities remained stable during maturation (Barbieri et al., 2007). Likewise, we could not find a link between the maturity and bacterial community composition of *T. aestivum* (Splivallo et al., 2019). In the case of *T. aestivum*, this discrepancy might be related to the special life cycle (several life cycles within a year) with unclear truffle fruiting season of this species (Büntgen et al., 2017).

Site-specific determinants shaped the composition of truffle microbiome

In our study, we demonstrated that factors shaping bacterial communities influenced the two orchards differently, with spatial location within the orchard being the main driver in Swiss and collection season in French orchard. This discrepancy might be explained by the expected differences in soil characteristics and climatic conditions in different truffle orchards. As truffles recruit their microbiome from the surrounding soil, soil

properties such as microbial community, pH, nutrients, and moisture are likely to influence the assemblage of truffle microbiome. In fact, it has been shown that soil communities and environmental factors affect the structure of the root microbiome (Colin et al., 2017; Zarraonaindia et al., 2015).

Overall, our data highlighted that the ecological drivers shaping the composition of bacterial community in truffle fruiting bodies are site-specific. Moreover, in contrast to other *Tuber* species, neither genotype nor maturity contributed in shaping the bacterial community composition in *T. aestivum*. Nevertheless, other described factors (i.e. collection season, and spatial location) could explain about 20% of the variability between communities, meaning that other involved factors remain to be discovered. Furthermore, large-scale studies would be required for a better understanding of the selection process of the microbial community by truffle primordia during the fruiting body formation at a microscopic scale.

Involvement of truffle microbiome in the human-sensed truffle aroma

Microbiomes have an essential role in aroma production in several food products. In fermented food products, for instance, the creation of the aroma compounds by lactic acid bacteria and/or yeasts (i.e. *Saccharomyces* and non-*Saccharomyces*) are the main drivers of overall aroma quality and consumer's preferences (Smid and Kleerebezem, 2014; Belda et al., 2017).

Truffles are appreciated and possess a high demand mainly owing to their unique and attractive aromas which might be partially derived from its microbiome (Splivallo et al., 2011, 2015; Vahdatzadeh et al., 2015, 2019). Indeed, the origin of many of these odorants have remained cryptic as they might be produced by truffle mycelium or/and associated microbes (Vahdatzadeh et al., 2015). The involvement of truffle microbiome in aroma formation has been mainly based on speculation. Buzzinie et al. (2005) demonstrated the production of some sulfur volatiles by yeasts isolated from fruiting bodies of the black and the white truffle (*T. melanosporum* and *T. magnatum*). Since sulfur volatiles are the most important and characteristic groups of truffle odorants, these authors hypothesised the possible contribution of truffle-associated yeasts in truffle aroma (Buzzini et al., 2005). Similarly, lack of some fruiting body key odorants in axenic mycelial cultures of *T. borchii* was used as indirect evidence for the involvement

of truffle microbiome in truffle aroma formation (Splivallo et al., 2007b; Tirillini et al., 2000; Vahdatzadeh and Splivallo, 2018). However, genome-based data of several *Tuber* species suggest that truffles encode for all the required genes for their aroma production (Martin et al., 2010; Murat et al., 2018b). Nonetheless, the function of the genes has never been confirmed and remains hypothetical (Martin et al., 2010; Splivallo et al., 2011; Murat et al., 2018b). Mainly genomic data provide limited information in two ways. First, the pathways leading to odorants, such as the Ehrlich pathway (chapter 1), are highly conserved in bacteria, yeast, as well as fungi. Second, the biosynthetic pathways of most of the odorants are unknown (i.e. formation of thiophene compounds). The role of the microbiome in aroma formation has only been elucidated in a single case of thiophene derivatives, characteristic of *T. borchii*, which are produced from unknown precursors by bacteria inhabiting the fruiting bodies but not by truffle (Splivallo et al., 2015).

In chapter 2 of this thesis, we speculated the origin of truffle odorants and the potential involvement of bacteria in production of these compounds using a meta-analysis approach, by combining a database on microbe-produced odorants (mVOC) (Lemfack et al., 2014) together with data on fungal volatiles (Chiron and Michelot, 2005). Our findings suggest that common truffle odorants (i.e. dimethyl sulfide, dimethyl disulfide, 1-octen-3-ol) might be produced by both truffles and many microbes (mixed origin), while species-specific truffle odorants (i.e. thiophene volatiles, 2,4-dithiapentane) might be produced by either fungi or microbes (Vahdatzadeh et al., 2015). Nevertheless, discerning the contribution of microbiome and truffle fungi, as well as proving the origin of truffle odorants, may require further investigations and creative thinking, since to date microbe-free fruiting bodies are unavailable. Furthermore, it requires the identification of aroma precursors of specific truffle odorants and the elucidation of their biosynthetic pathways in truffle fruiting bodies.

Potential role of the truffle-associated microbiome in aroma deterioration upon storage

Deterioration involves any changes in food (i.e. appearance, texture, flavour and aroma) resulting from microbial growth, oxidation, and dehydration, which influence consumer acceptance (Hayes, 1995).

Truffles are highly perishable culinary delicacies with a short shelf life of 1-2 weeks. Studies on post-harvest storage highlighted that truffle aroma changes deeply upon storage (Bellesia et al., 1998; Falasconi et al., 2005; Aprea et al., 2007; Culleré et al., 2013b; Pennazza et al., 2013; Splivallo et al., 2015). Storage period and conditions such as temperature and several preservation techniques (i.e. sterilisation, freezing, freeze-drying, drying, canning, gamma radiation, and modified atmosphere packaging) have been reported to influence truffle aroma (Nazzaro et al., 2007; Saltarelli et al., 2008; Reale et al., 2009; Rivera et al., 2010a, 2011b, 2011a; Culleré et al., 2012, 2013b; Palacios et al., 2014; Campo et al., 2017; Vahdatzadeh et al., 2019). Besides influencing the aroma, storage affects the composition of truffle-associated microbial communities. However, few studies have investigated this and only employing culture-dependent methods (Saltarelli et al., 2008; Rivera et al., 2010a).

Spoilage is mainly due to microbes, yet microbes that naturally colonise truffle fruiting bodies might also contribute to truffle smell. Hence, in chapter 4, we investigated the influence of truffle microbiome (employing a culture-free method) on aroma deterioration of *T.aestivum* upon post-harvest storage. (Vahdatzadeh et al., 2019).

Truffle´s endemic microbiome replaced by food spoilage bacteria during storage

In fresh fruiting bodies, the truffle-associated microbiome mirrored the previous result from the same species (Splivallo et al., 2019) and were dominated mainly by α-Proteobacteria and in few cases by β-Proteobacteria or Sphingobacteria (Vahdatzadeh et al., 2019). Our data indicated the gradual shift of the endemic truffle bacteria to detrimental bacteria, mainly belonging to the γ-Proteobacteria class (i.e. *Enterobacteriaceae* family), and to a lesser extent by members of the Bacilli class (i.e. various families affiliated to lactic acid bacteria) upon storage, regardless of the initial

diversity of the bacterial classes (Vahdatzadeh et al., 2019). The *Enterobacteriaceae* and lactic acid bacteria are well-known food spoilage bacteria reported earlier in truffles (Nazzaro et al., 2007; Reale et al., 2009; Rivera et al., 2010a, 2010b, 2011a, 2011b) as well as in other foods (Blackburn, 2006; Lim et al., 2014). Interestingly, some of the prevalent genera of detrimental bacteria of truffle, such as *Serratia*, have been reported to alter the food aroma quality and cause off-odour during storage in other food products including meat, coffee beans (Gallois and Grimont, 1985; Hernández-Macedo et al., 2011). Changes induced by storage were not only in the structure of bacterial communities but also in the bacterial density, which rapidly increased by 4-30 fold upon storage (Vahdatzadeh et al., 2019). This corroborated earlier results on the cultivable fraction of bacteria of several *Tuber* species (*T. borchii*, *T. melanosporum*, *T.aestivum*) stored at 4°C after 15 days (Saltarelli et al., 2008). Several studies attempted to prolong the truffle shelf life by limiting the bacterial growth using post-harvest preservation techniques such as sterilisation or gamma-ray irradiation. However, based on our data, shelf life prolongation might be attainable by new preservation techniques which preserve the endemic truffle microbiome.

Storage profoundly impacts truffle aroma quality

Similar to the bacterial density, the total number of emitted volatiles showed a strong increase in stored truffles. Our findings demonstrated an important shift in the volatile profile of *T. aestivum* upon storage. Also, the existence of the five freshness volatile markers (volatiles that decreased in concentration during storage) including dimethyl sulfide (truffle/sulfurous (Culleré et al., 2010)), 2-butanone (ethereal/camphor-like (Garg et al., 2018)) and twelve spoilage markers (volatiles that increased in concentration during storage)) such as 2- and 3-methylbutanol (fermented/fusel (Schmidberger and Schieberle, 2017)), and 2-phenylethanol (rose-like (Splivallo and Culleré, 2016)) were illustrated. Many of these volatiles are key contributors to the human-sensed truffle aroma (Culleré et al., 2010; Liu et al., 2013; Splivallo and Ebeler, 2015; Schmidberger and Schieberle, 2017). Since some of these freshness/spoilage volatile markers have been previously reported during storage or preservation techniques from several truffle species, they might be considered as universal

freshness/spoilage markers in truffles (Bellesia et al., 1998, 2001; Aprea et al., 2007; Culleré et al., 2012; Palacios et al., 2014). We further suggested that changes in the odorants of stored truffles might be due to the decrease of freshness markers and the emergence of spoilage markers (Vahdatzadeh et al., 2019). Nevertheless, demonstrating the contribution of specific volatiles requires further sensory studies and determination of OAVs.

Spoilage bacteria might be responsible for the aroma loss in stored truffles

As previously discussed (chapter 2), microbes might be partially responsible for the fresh truffle aroma (Vahdatzadeh et al., 2015). The data presented in chapter 4, put forward the hypothesis of mixed origin (bacteria and fungi) of truffle aroma by illustrating a strong correlation between volatile markers and specific bacterial classes. For instance, freshness markers such as dimethyl sulfide, 2- butanone, and ethyl acetate were correlated with the dominant bacterial classes in the fresh truffle (commensal microbiome), whereas spoilage markers were correlated with the presence of the Bacilli (detrimental microbiome) (Vahdatzadeh et al., 2019). Interestingly, production of spoilage markers by many bacterial classes, among them the Bacilli class, have been reported elsewhere (Lemfack et al., 2014). Also, correlations were observed among C_8 volatiles including 3-octanone, 1-octanol and 2-octenol and major class of detrimental microbiome γ-Proteobacteria. This correlation suggests the microbial origin of these volatiles, similar to what has been observed in the fungus *Mortierella elongate* (Uehling et al., 2017), or a mixed origin by both truffle and microbes (Vahdatzadeh et al., 2019).

In general, our results highlighted the profound effect of storage on both truffle aroma and microbiome (shift from commensal to detrimental bacteria) and indicated the existence of freshness and spoilage volatile markers. They also suggested a possible involvement of commensal/detrimental bacteria in fresh truffle aroma/aroma deterioration upon storage. These findings should permit a better evaluation of truffle quality and possibly design new preservation techniques. Nonetheless, to acquire a more comprehensive view of the storage process, spoilage fungi need to be also monitored since they might also be involved in aroma changes.

5 Conclusions

The results of this thesis provide unprecedented insight into the influence of truffle genotype and microbiome on truffle aroma. This study concludes that truffle genotype has a profound influence on truffle aroma variability as well as on the human aroma perception. Our data also highlight that the structure of the endemic microbiome in fresh truffles is mainly dominated by the α-Proteobacteria class and in fewer cases, the β-Proteobacteria and Sphingobacteria classes. They further illustrate that factors shaping the structure of bacterial communities are site-specific. In contrast to other fungi, truffle genotype and the degree of the fruiting body maturity seem not to contribute to driving the assembly of truffle microbiome. Finally, the results of this thesis also suggest the involvement of microbes in the formation of the human-sensed truffle aroma as well as aroma deterioration upon post-harvest storage.

These findings can be promising in two aspects of the truffle industry. Firstly, instead of spore infestation in nurseries, production of truffles with higher consumer acceptance might be achieved through the inoculation of seedlings with mycelial strains associated with more desired aromas. Secondly, truffle shelf life might be prolonged, and its aroma can be possibly maintained through the conservation of the native microbiome by applying targeted preservation techniques.

Overall, our results demonstrate the need for a better understanding of the recruitment process of diverse microbial communities by truffle fruiting bodies, and the ecological functions of the truffle microbiome, especially in aroma formation. Moreover, elucidation of the origin of truffle odorants and their biosynthetic pathway might pave the way toward natural truffle aroma production with higher customer acceptability.

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7 Publications

7.1 Chapter 1: Improving truffle mycelium flavour through strain selection targeting volatiles of the Ehrlich pathway

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MV: 50%; RS: 50%

(2) Conducting tests and experiments

MV: 100%

(3) Compilation of data sets and figures

MV: 90%; RS: 10%

(4) Analysis and interpretation of data

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Improving truffle mycelium flavour through strain selection targeting volatiles of the Ehrlich pathway

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Truffles (*Tuber* spp.) are the fruiting bodies of symbiotic fungi, which are prized food delicacies. The marked aroma variability observed among truffles of the same species has been attributed to a series of factors that are still debated. This is because factors (i.e. genetics, maturation, geographical location and the microbial community colonizing truffles) often co-vary in truffle orchards. Here, we removed the co-variance effect by investigating truffle flavour in axenic cultures of nine strains of the white truffle *Tuber borchii*. This allowed us to investigate the influence of genetics on truffle aroma. Specifically, we quantified aroma variability and explored whether strain selection could be used to improve human-sensed truffle flavour. Our results illustrate that aroma variability among strains is predominantly linked to amino acid catabolism through the Ehrlich pathway, as confirmed by ^{13}C labelling experiments. We furthermore exemplified through sensory analysis that the human nose is able to distinguish among strains and that sulfur volatiles derived from the catabolism of methionine have the strongest influence on aroma characteristics. Overall, our results demonstrate that genetics influences truffle aroma much more deeply than previously thought and illustrate the usefulness of strain selection for improving truffle flavour.

Truffles (*Tuber* spp.), fruiting bodies of symbiotic fungi which develop underground, are well known for their enticing and captivating aromas[1]. They are edible fungal-organ bearing spores, which result from sexual reproduction[2–4]. Indeed, truffles are heterothallic organisms, meaning that they need two mating types (maternal and paternal) for reproduction. Mating occurs once the maternal mating type that colonizes plant roots in a symbiotic organ known as ectomycorrhizas encounters an individual of opposite (paternal) mating type. This results in the formation of a haploid fruiting body containing spores of both mating types[4]. Molecular based data suggest the existence of about 180 truffle species in various regions of the world[5], of which about 30 are commercially traded. Prices range from a few hundred Euros per kg for the cheapest truffle species up to thousands of Euros for the most expensive ones such as the white Piedmont truffle *Tuber magnatum* or the Périgord black truffle *Tuber melanosporum*.

Truffle fruiting bodies are considered a food delicacy, mostly due to their unique aromas[1]. Volatiles responsible for their distinctive smells are a blend of alcohols, ketones, aldehydes, aromatic and sulfur compounds. As with any other food products[6], only a small fraction of all volatiles emitted by truffles (the so-called odorants) are responsible for the smell perceived by humans[7–9]. In terms of composition, certain odorants are common to many truffle species while others are species specific or limited to a few species only. For example, 2-methylbutanal, 3-methylbutanal, 2- methylbutan-1-ol, 3-methylbutanol and oct-1-en-3-ol are common to most truffle species[10] while 2,4-dithiapentane and 3-methyl-4,5-(2 H)thiophene have been exclusively described in fruiting bodies of the white species *T. magnatum*, and *Tuber borchii*, respectively[7,11,12]. The volatile composition can vary throughout the various stages of a truffle's life cycle. This is for instance illustrated by 3-methyl-4,5-(2 H)thiophene that seems to be exclusively emitted during the sexual stage (fruiting bodies) of *T. borchii*. This compound is indeed not detectable from axenic mycelial cultures, even when they are re-inoculated with bacteria that produce this volatile in fruiting bodies[12]. Overall, the volatile profile of axenic cultures of truffle mycelium tends to be less complex in terms of the number of compounds than one of truffle fruiting bodies[13,14]. This disparity is possibly caused by differences in developmental stages or the lack of interacting microbes as highlighted hereafter[15].

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Truffle fruiting bodies are heavily colonized by bacteria and to a lesser extent by yeasts, filamentous fungi and viruses[15]. The origin of many truffle odorants is hence unclear, as they might be synthesized by the truffle itself or by its microbiome. The contribution of microbes to truffle aroma has indeed only been demonstrated in a single case, which links bacteria inhabiting the fruiting body of *T. borchii* to the production of thiophene derivatives (i.e. 3-methyl-4,5-(2 H)thiophene)[7,12]. Additionally, indirect evidence suggests that bacteria might be exclusively responsible for the emission of 2,4-dithiapentane in *T. magnatum* as well[15]. By contrast, the origin of odorants that are more common among truffles (i.e. dimethyl sulfide, 2- methylbutan-1-ol and 3-methylbutan-1-ol and their aldehydes derivatives, just to cite a few examples) remains elusive since, theoretically, they may be synthesized by both truffles and their microbiomes[15].

Uncertainties currently exist not only about the origins of truffle odorants, but also about their precursors. Indeed, the identity of aroma precursors in truffles is to a large extent based on speculation and indirect evidence. Following the sequencing of the genome of the black truffle *T. melanosporum*[16], it has been suggested that numerous truffle odorants were produced from amino acid catabolism through the Ehrlich pathway. In this pathway, an amino acid is first deaminated into an α-keto acid, followed by a decarboxylation into an aldehyde and either a reduction or oxidation into an alcohol or acid, respectively[10,16,17]. In this way, leucine, isoleucine, phenylalanine and methionine are respectively transformed into 3-methylbutanal, 2-methylbutanal, 2-phenylacetaldehyde and 3-methylsulfanylpropanal and their corresponding alcohols and acids[17]. Supplying axenic cultures of truffle mycelium with leucine, isoleucine, phenylalanine and methionine was shown to induce numerous volatiles of the Ehrlich pathway[18–20], suggesting that these amino acids were either the direct precursors of those volatiles or indirectly induced them. Nevertheless, demonstrating the existence of the Ehrlich pathway beyond a reasonable doubt shall ultimately require feeding experiments with isotopically-labelled amino acids, which has not been performed to date.

Differences in the aroma profiles of truffles do not only exist among species and/or developmental stages as highlighted earlier, but also within truffles of the same species. Indeed, a major variability in the concentration of four and eight carbon-containing volatiles (i.e. oct-1-en-3-ol and 2-butanone) has been documented for *Tuber aestivum* fruiting bodies collected a few centimeters apart in the same truffle orchards[21,22]. This aroma variability has been linked to genetic differences[21,22]. Considering that strain selection has been successfully performed in microbes to improve the final flavour of fermented food products (i.e. cheese and wine) or to eliminate off-flavour compounds[23–26], a similar approach might improve the characteristics of truffle flavour produced through axenic truffle mycelium cultures.

The main aim of this study was to assess whether strain selection could be used for improving human-sensed truffle flavour produced through mycelial fermentation. To answer this, nine strains of the white truffle *T. borchii* were tested in various feeding experiments and sensory tests (see Fig. 1 for the experimental design). This species, endemic to Europe and introduced in New Zealand[5] was chosen because of the good growth of its mycelium compared to other truffle species[27]. Specifically, in the first set of experiments, the extent of aroma variability among strains was assessed and volatiles responsible for this variability were identified. Subsequently, the existence of the Ehrlich pathway was tested with isotopically labelled amino acids and finally, sensory tests were performed to assess whether the human nose was capable of differentiating among strains.

Results

T. borchii mycelia vary in their volatile profiles. The aim of this first experiment was to assess the variability in aroma profiles of mycelial cultures of *T. borchii*. For this purpose, the aroma profiles of nine strains (axenic cultures grown in malt extract) and one fruiting body (included here for completeness) of *T. borchii* (Table 1) were analysed by solid-phase microextraction gas chromatography-mass spectroscopy (SPME-GC/MS). Volatile profiles, generated for three independent replicates of all strains/fruiting body, were processed for peak realignment with the Tagfinder software[28]. This resulted in a data matrix of specific TAGs (volatiles) in each sample (Supplementary Table S1 illustrates the raw data of TAGs normalized to the total ion current (TIC)). Principle Component Analysis (PCA) applied to the volatile profile in Supplementary Table S1 could explain 59% of the data variability as seen in Fig. 2A, highlighting differences among strains.

TAGs which significantly differed in concentrations among strains were identified using the non-parametric Kruskal-Wallis statistical test performed in R[29]. Those TAGs could be assigned to 29 compounds, which are represented in a heat map in Fig. 2B. These include one sulfur-containing volatile, alkenes, alcohols, aldehydes, ketones, aromatic compounds and eight unidentified volatiles. Both qualitative and quantitative differences were detected among strains. Interestingly, one-third of the volatiles that varied in concentrations among strains might be products of the Ehrlich pathway. Common volatiles produced by most *T. borchii* strains included 2- and 3-methylbutanal, benzaldehyde, 2-phenylethan-1-ol whereas other volatiles such as 2-phenylacetaldehyde, 2- and 3-methylbutan-1-ol and 2-methylpropan-1-ol were specific to two or three strains and 3-methylsulfanylpropanal was detected from a single strain (strain 2) only (Fig. 2B).

Our data demonstrated that the largest part of aroma variability among strains was due to quantitative differences in volatiles possibly derived from amino acids catabolism (Ehrlich pathway).

Confirming the existence of the Ehrlich Pathway in _T. borchii_ through feeding mycelia with ^{13}C labelled amino acids. Considering the important variability in volatiles possibly derived from amino acid catabolism, we tested the existence of the Ehrlich pathway in truffles. With this aim in mind, three mycelial strains (strains 2, 3 and 5) were supplied with four amino acids separately, namely leucine, isoleucine, phenylalanine, and methionine (unlabelled and ^{13}C labelled) and their aromas were profiled by pressure-balanced headspace-GC/MS. Pressure-balanced headspace extraction and subsequent trapping on a charcoal cartridge was chosen in favour of SPME for its improved reproducibility. In all strains, leucine induced 3-methylbutanal and 3-methylbutan-1-ol; isoleucine: 2-methylbutanal and 2-methylbutan-1-ol; phenylalanine, 2-phenylacetaldehyde,

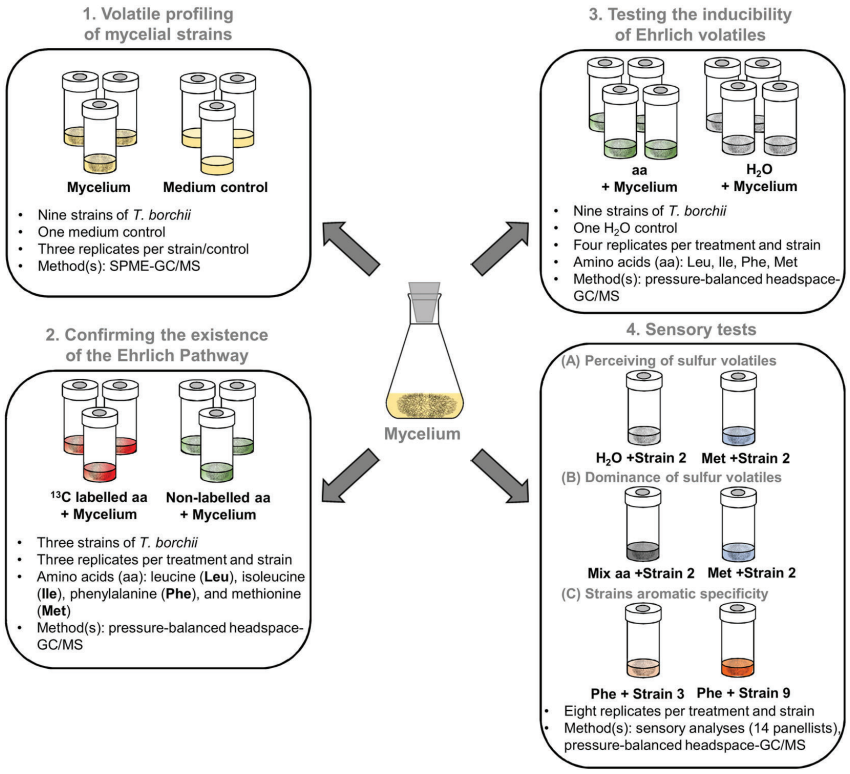

Figure 1. Experimental design. This figure highlights the number of replicates used for each experiment and the techniques employed.

Mycelium strain or fruiting body	Accession number (GenBank - NCBI)	Accession number reported in	Origin
Strain 1	DQ679802	Bonuso *et al.*[53]	Emilia-Romagna, Italy
Strain 2	FJ554505	Bonuso *et al.*[53]	Emilia-Romagna, Italy
Strain 3	FJ554476	Bonuso *et al.*[53]	Emilia-Romagna, Italy
Strain 4	MF686459	Current work	Piedmont, Italy
Strain 5	KP244305	Splivallo *et al.*[7]	Piedmont, Italy
Strain 6	KF414978	Splivallo *et al.*[12]	Piedmont, Italy
Strain 7	KP244306	Splivallo *et al.*[7]	Piedmont, Italy
Strain 8	KP244307	Splivallo *et al.*[7]	Piedmont, Italy
Strain 9	MF686460	Current work	Canterbury, New Zealand
Fruiting body*	—	—	Piedmont, Italy

Table 1. *T. borchii* strains and their origins. *Identified based on spores' morphology.

2-phenylethan-1-ol, and benzaldehyde; methionine, 3-methylsulfanylpropanal, dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS) (Fig. 3). Comparison of the mass spectra of all three mycelial strains supplemented with ^{13}C labelled and unlabelled amino acids gave comparable results for all strains and showed that all the labelled carbon atoms were fully incorporated in target volatiles (Fig. 3 and Supplementary Fig. S1), hence confirming the existence of the Ehrlich pathway in *T. borchii*.

Testing the induction of Ehrlich volatiles in *T. borchii*. Having confirmed the existence of the Ehrlich pathway in truffles, and documented an important concentration variability in resulting volatiles, we next questioned whether strains differed in their ability to produce those volatiles in the presence of amino acids. With this aim, nine mycelial strains were supplemented with non-labelled amino acids (leucine, isoleucine,

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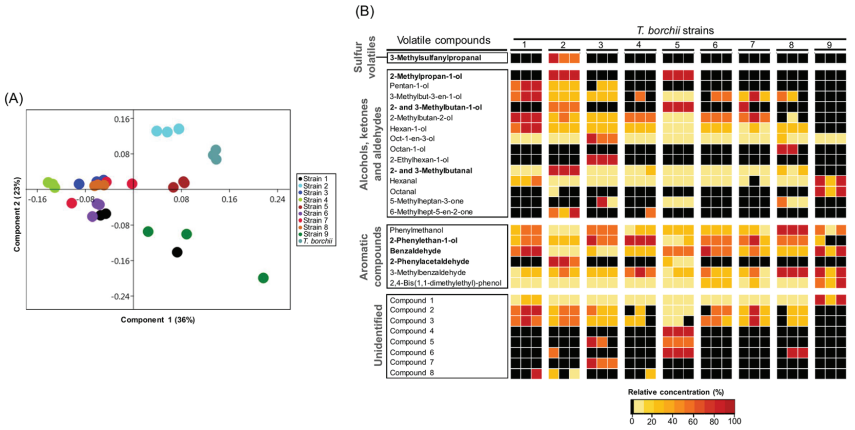

Figure 2. Volatile profiles of nine *T. borchii* mycelial strains. (**A**) PCA (based on Supplementary Table S1) illustrates the variability in volatile profile for nine *T. borchii* mycelial strains and one fruiting body (n = 3 replicates per sample). (**B**) The heatmap highlights volatile compounds which concentrations significantly differed among strains (n = 3 replicates per strain, $p < 0.05$, Kruskal-Wallis test with $\alpha = 0.05$). Volatile compounds derived from the Ehrlich pathway are highlighted in bold.

phenylalanine, and methionine) and the concentration of induced volatiles was compared to the one produced by un-supplemented strains. For illustration, chromatograms of strains 5 and 7, both supplemented with Met and unsupplemented (water control) is shown in Fig. 4. Strain 5 displayed a considerably higher level of volatiles induction (DMDS, 3-methylsulfanylpropanal and DMTS) compared to strain 7. Quantifying volatiles in all samples highlighted that supplementing amino acids induced the production of volatiles to different extents depending on the strain. In Fig. 5, statistical differences in the concentrations of nine volatiles among supplemented and unsupplemented samples are indicated above bars when significant (i.e. 3-methylbutanal was 152 times higher in strain 2 supplemented with leucine compared to the unsupplemented H_2O sample). Comparing relative concentrations among samples that emitted the highest or lowest concentration of a specific volatile compound similarly highlights statistical differences (i.e. 3-methylsulfanylpropanal was induced 41 times: strain $2_{MAX} = 1.46 \pm 0.67$, strain $1_{MIN} = 0.04 \pm 0.03$, $p = 0.02$, Kruskal-Wallis test with $\alpha = 0.05$); 2-phenylacetaldehyde was induced 55 times: strain $3_{MAX} = 26.99 \pm 1.73$, strain $6_{MIN} = 0.48 \pm 0.09$, $p = 0.02$, Kruskal-Wallis test with $\alpha = 0.05$). Altogether, a high variability in the production of volatiles compounds derived from the Ehrlich pathway was observed among strains upon amino acid addition, resulting in inductions of up to 1327 times compared to control samples (3-methylbutanal, strain 9).

Sensory tests with *T. borchii* mycelial strains. Considering the differences in aroma profile exemplified among strains of *T. borchii* in our earlier experiments, we questioned if these differences would impact human-sensed aroma perception. Indeed, to have a possible impact on the overall aroma, a volatile compound needs to be detectible by the human nose (some volatile compounds are odourless) and be present above its detection threshold. A total of three sensory tests were performed to address various questions. The first test was carried out to investigate the ability of the human nose to perceive induced volatiles in *T. borchii* strains upon amino acid supplementation. A second test aimed at exploring the capability of the human nose to differentiate between aromas induced by a single amino acid or a mixture using a single strain. In the third test, the ability of the human nose to distinguish among strains supplemented with the same amino acid was examined. All tests were performed by 14 panellists and consisted of a triangle test (discrimination test) and an assessment of aroma attributes as described in the material and methods section.

Induced sulfur containing volatiles are perceived by the human nose. In the first test, mycelial strain 2 was supplemented with methionine or water (control). The result of the triangle test illustrated that the panellists were able to distinguish between unsupplemented and methionine supplemented samples ($p = 0.02 < \alpha$, one-sided binomial proportions test with $\alpha = 0.05$), hence confirming that sulfur containing volatiles (hereafter "sulfur volatiles") of the Ehrlich pathway were perceived by the human nose.

In terms of aroma attributes, differences between samples could be ascribed to sulfur and fermented/roasted notes that were highest in methionine supplemented samples and floral notes that were the highest in unsupplemented samples (Fig. 6A). Samples also differed in terms of intensity with methionine supplemented samples being the most intense (Fig. 6A). The samples were further analysed by pressure-balanced headspace-GC/MS to relate volatile profiles to the aroma impressions of the panellists (Fig. 6A). The marked sulfurous/garlicky notes reported by the panellists in the methionine supplemented sample, corresponded to the induction of sulfur volatiles, whereas the flowery notes of the unsupplemented sample corresponded to the induction of 2-phenylacetaldehyde and 2-phenylethan-1-ol.

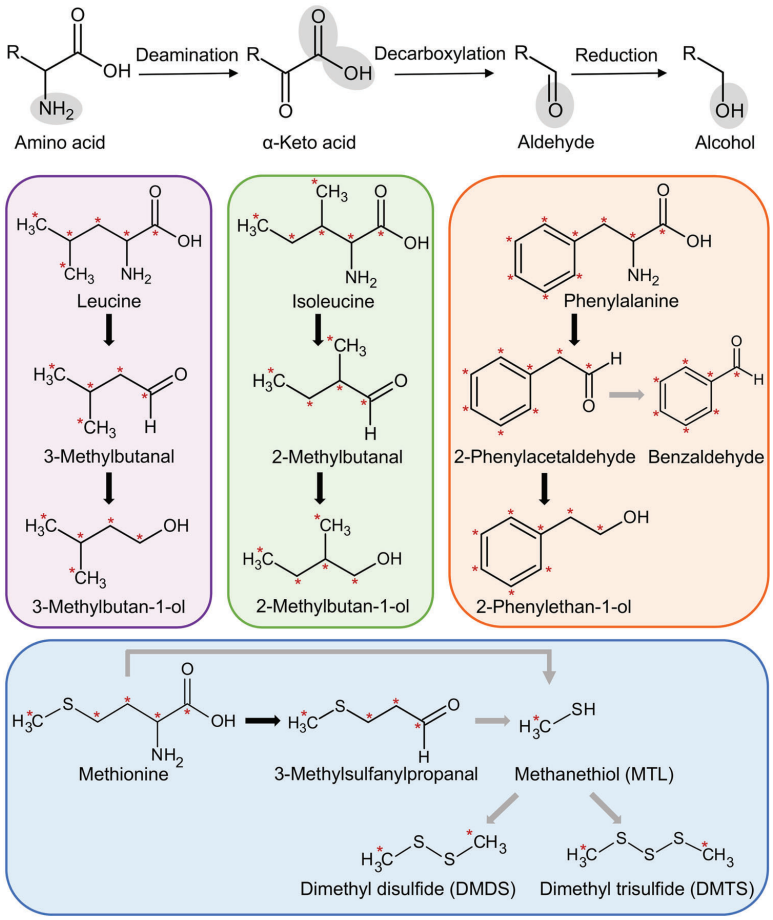

Figure 3. Amino acids catabolism in *Tuber borchii*. The scheme on top illustrates the three steps of the Ehrlich pathway. Specific structures of amino acids and volatile compounds detected in our experiments (with the exception of MTL which was not detected but is shown here for completeness) are shown below with ^{13}C label incorporation. Labelling experiments with three mycelial strains (strains 2, 3 and 5) gave comparable results. Refer to Supplementary Fig. S1 for details.

Induced volatiles by single or mixture of amino acids are not distinguished by the human nose. A second triangle test was performed to estimate the overall importance of sulfur volatiles compared to other (non-sulfur) aroma compounds. Essentially, the aromas induced in a single strain (strain 2 as used in the first trial) by methionine were compared to those induced by a mixture of amino acids that included methionine and also leucine, isoleucine and phenylalanine. The triangle test of the second trial illustrated that the human nose was unable to distinguish among samples supplemented with methionine only compared to those supplemented with a mixture of amino acids (that included methionine as well) ($p = 0.86 > \alpha$, one-sided binomial proportions test with $\alpha = 0.05$). Similarly, no significant difference in overall aroma intensity among the samples was detected (Fig. 6B). However, quantification of volatiles by pressure-balanced headspace-GC/MS highlighted the marked differences in DMDS and benzaldehyde, that were significantly higher in the sample supplemented with the amino acid mixture (Fig. 6B).

The human nose can differentiate among strains. A third triangle test was performed to examine the ability of the human nose to distinguish between strains 3 and 9 supplemented here with phenylalanine only.

The triangle test results demonstrated that the human nose could differentiate between strains supplemented with phenylalanine ($p = 0.004$, one-sided binomial proportions test, $\alpha = 0.05$). These findings were corroborated by the sensory test since higher floral notes were attributed to strain 3 (Fig. 6C). Profiling of volatiles revealed

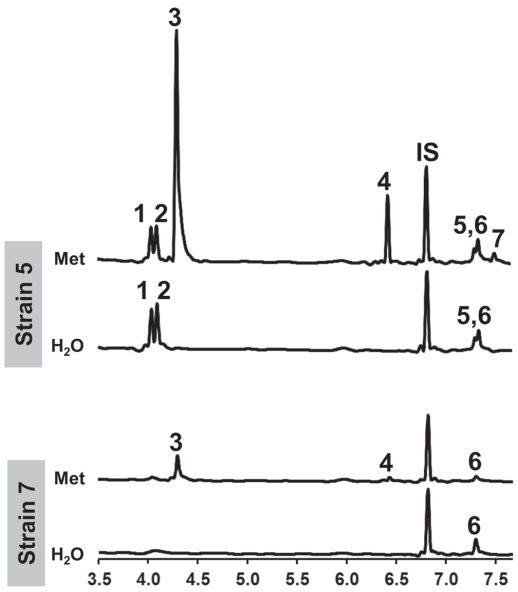

Figure 4. Sulfur volatile compounds induced by methionine in *T. borchii*. Chromatograms are shown for two strains supplemented with Met (20 mM) or water (control). The y-axis is comparable for all chromatograms. Note the stronger S-volatile induction of strain 5 compared to strain 7. Volatile compounds: **(1)** 3-methylbutan-1-ol; **(2)** 2-methylbutan-1-ol; **(3)** DMDS; **(4)** 3-methylsulfanylpropanal; **(5)** benzaldehyde; **(6)** unidentified; **(7)** DMTS and IS (internal standard).

that the concentrations of six volatiles significantly differed among strains 3 and 9, with 2-phenylacetaldehyde, 2-phenylethan-1-ol and benzaldehyde being the highest in strain 3 ($p < 0.05$, Kruskal-Wallis test with $\alpha = 0.05$) (Fig. 6C).

Discussion

Genetics is a powerful tool to improve flavour and aroma of food products and/or eliminate off-flavours[23–26]. The present study demonstrated how strain selection could be used to improve truffle mycelium flavour. Type of strain however is not the only means of flavour improvement in truffles since Xiao *et al.*[20] illustrated that desirable sulfurous, mushroom and earthy aromas could also be induced in the black truffle *T. melanosproum* by repeated freeze-thaw cycles.

The aroma emitted by axenic cultures of three truffle species (*T. borchii*, *T. melanosporum*, *T. formosanum*) have been described earlier by various groups[13,14,19,20,30,31], yet none of those studies investigated aroma variability within the same species. Here, we used nine strains of *T. borchii* for the latter purpose. Of the 21 volatiles identified from *T. borchii* mycelia grown on malt extract (Fig. 2B), only five have been previously reported from two *T. borchii* strains including 3-methylbutan-1-ol, oct-1-en-3-ol, 2-phenylethan-1-ol, phenylmethanol and 2,4-bis(1,1-dimethylethyl)-phenol[13,14]. Tirillini *et al.*[14] could nevertheless detect 28 additional volatiles including dimethyltrisulfide through dynamic headspace sampling. Similarly, ten volatiles reported in Fig. 2B have been described from axenic cultures of *T. melanosporum* or *T. formosanum*, which also produced numerous volatiles not reported here[30,31]. These similarities and discrepancies in volatile emission among our results and literature might be attributed to various factors. For example, cultural conditions might have a major impact on fungal volatiles[32,33]. In the case of axenic cultures of different truffle species (*T. melanosporum*, *T. sinense*, *T. indicum*, and *T. aestivum*), Tang *et al.*[34] demonstrated that cultural conditions indeed had a stronger influence on the composition of emitted volatiles than species itself. Besides media composition, volatile sampling techniques might also influence volatile profiles[35], and so does sample processing (i.e. freezing)[1,20,36]. Nevertheless, considering that in our study, all strains of *T. borchii* were handled in the same way, the aroma variability observed among strains (Fig. 2A,B) was without a doubt attributed to genetic differences among strains.

The influence of genetics on truffle aroma had been reported earlier in *T. aestivum*[21,22] fruiting bodies for specific groups of volatiles, namely four (C_4) and eight (C_8) carbon-containing compounds (i.e. C_8: oct-1-en-3-one, oct-1-en-3-ol and t-2-octenal; C_4: butan-2-one and butan-2-ol). Our data support the influence of genetics on the 29 volatiles reported in Fig. 2B, and particularly on volatiles of the Ehrlich pathway, which could not be shown in earlier work by our group[21,22]. This apparent contradiction between our earlier results and those presented here can be explained by differences in study systems (axenic mycelial cultures versus fruiting bodies). Indeed, fruiting bodies are heavily colonized by microbes (i.e. bacteria and yeasts), which, in addition to

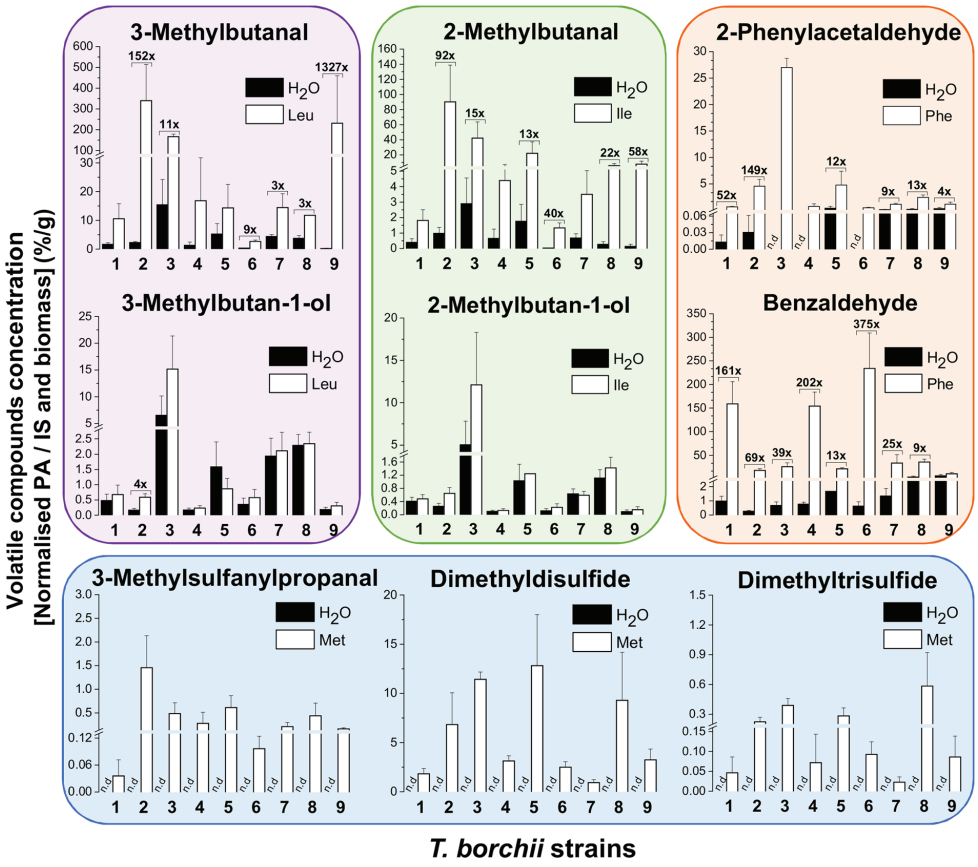

Figure 5. Induction of volatiles compounds derived from the Ehrlich pathway in *T. borchii*. Bars represent the normalised concentrations (average ± standard error for four biological replicates) of volatile compounds in nine strains of *T. borchii* – either unsupplemented (H_2O) or supplemented with 20 mM amino acids namely leucine (violet), isoleucine (green), phenylalanine (orange), and methionine (blue). Concentrations of specific volatile compounds that differed between supplemented and unsupplemented strains are indicated above bars (as the "induction" x) when statistically significant ($p < 0.05$, Kruskal-Wallis test with $\alpha = 0.05$).

truffles, are able to produce volatiles from the Ehrlich pathway[15]. The production of the Ehrlich-derived volatiles 2-methylbutan-1-ol, 3-methylbutan-1-ol along with some sulfur volatiles (3-methylsulfanylpropanal, dimethyl sulfide (DMS), DMDS and DMTS) have been reported from yeasts directly isolated from truffle fruiting body[37]. Besides microbes, other factors might influence fruiting body aroma (i.e. maturation[38], geographical location[39]). Many of those factors often co-vary (i.e. genetics and geography), thus using fruiting bodies for investigating the factors behind aroma variability in truffles can only lead to limited conclusions.

The Ehrlich pathway, first described in the budding yeast *Saccharomyces cerevisiae*, consists of a three-step process, in which an amino acid is first deaminated into a α-keto acid, followed by a decarboxylation into an aldehyde and a reduction or oxidation into an alcohol or acid[17]. We demonstrated here the existence of this pathway in *T. borchii* using amino acids with ^{13}C labelled carbons, yet, proving the origin of non-carbon atoms (i.e. sulfur, oxygen) was beyond the scope of our study. According to the "conventional Ehrlich pathway", phenylalanine leads to two volatiles, 2-phenylacetaldehyde and 2-phenylethan-1-ol[17]. In our data (Fig. 3), the incorporation of ^{13}C labels into a third volatile (benzaldehyde) hints at possible pathway differences between *Saccharomyces* yeasts and truffles. Considering that benzaldehyde might be produced from phenylalanine through both enzymatic and chemical reactions in some bacteria[40], suggests that this might similarly occur in truffles. Identifying the exact pathway in truffles shall be the focus of further studies.

Methionine catabolism leads to the formation of sulfur volatiles through two hypothetical pathways for which candidate enzymes have been identified in the genome of *T. melanosporum*[10,16]. Indeed, in the "standard" Ehrlich pathway, methionine might be converted into 4-methylthio-2-oxobutyric acid (KMBA) via

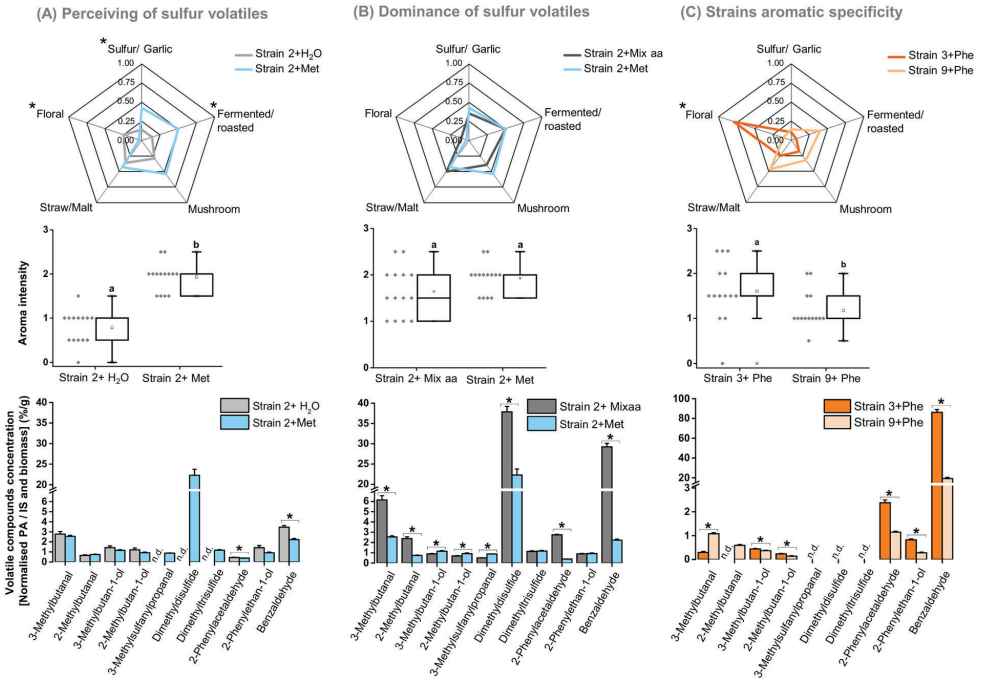

Figure 6. Results of sensory tests. From top to bottom, graphs illustrate aroma descriptors (spider chart), aroma intensities (box plot) and concentration of volatile compounds (bar chart), respectively. Refer to the main text for details. Statistical differences between samples (i.e. for each aroma descriptor or for aroma intensity or the concentration of specific volatile compounds) are marked with a star or with different letters (box plot) ($p < 0.05$, Kruskal-Wallis test with $\alpha = 0.05$).

transamination. Subsequently, KMBA decarboxylation might lead to 3-methylsulfanylpropanal which might further non-enzymatically decompose to methanethiol (MTL). The latter might also be produced from KMBA through both chemical and enzymatic reactions by C-S lyase enzymes (i.e. cystathionine β- and γ-lyases). In the alternative "non-standard" Ehrlich pathway, methionine might directly be converted into MTL by enzymatic reactions (i.e. methionine γ-lyase). In both standard and non-standard pathways, MTL can spontaneously oxidize to DMS, DMDS and DMTS[10]. The induction of 3-methylsulfanylpropanal observed in our data (Fig. 3) supports the existence of at least the standard Ehrlich pathway for methionine catabolism in *T. borchii*. By contrast, the absence of DMS, a typical sulfur volatile of many truffle species[10] from our mycelial cultures might be interpreted in two ways. On one side, DMS might not be induced by the cultural conditions used here, and different conditions might have favoured it as reported for *T. melanosporum* and *T. formosanum*[30,31]. On the other side, DMS in truffle fruiting bodies might actually be exclusively produced by bacteria (and not by the truffle mycelium), as demonstrated for some cyclical sulfur volatiles in *T. borchii*[12]. Either hypothesis will require a more detailed characterization of methionine catabolism pathway to be tested.

Volatiles produced through the Ehrlich pathway are well-known food odorants[6]. For example, taken as pure compounds, 2-phenylethan-1-ol has a rose-like odour, 3-methylbutan-1-ol is reminiscent of cheese and whiskey and sulfur volatiles (i.e. DMS, DMDS, DMTS and 3-methylsulfanylpropanal) smell like garlic, rotten food, and cooked potatoes[8,15]. Aroma perception of specific food depends on the interplay among odorants and on their respective concentrations[6], yet, typically, only a small fraction of all volatiles contribute to human-perceived aroma. Our sensory tests illustrated the importance of methionine in the formation of sulfur containing odorants in axenic truffle cultures. Indeed, sulfur volatiles are also major odorants in truffle fruiting bodies[7,8,15]. In our sensory tests, panellists predominantly used sulfur/garlicky and fermented/roasted notes to describe the aroma of mycelium supplemented with methionine. Even if not determined here, this strongly suggests that the concentration of sulfur volatiles were higher than their detection threshold (odour activity value (OAV) > 1) in methionine supplemented samples. This is further supported by the fact that sulfur volatiles tend to have much lower odour thresholds compared to other volatiles of the Ehrlich pathway (i.e. odour threshold in water (part per billion) of sulfur volatiles DMDS (0.16–12)[41–43] and DMTS (0.005–0.01)[41,43] versus non-sulfur volatiles such as 2-phenylethan-1-ol (750–1100)[44,45] and 3-methyl-1-butanol (250–300))[44,45].

Panellists could further differentiate among mycelial strains supplemented with the same amino acids. This illustrates that genetics does not only influence volatile production in truffles as highlighted earlier, but that those differences have an impact on the overall aroma characteristics. Similar findings of strains influence on aroma characteristics have been also demonstrated for strawberries and baker's yeast[46,47], among numerous other examples. Specifically, in domesticated strawberries, an insertional mutation was shown to result in flavour loss compared to wild strawberries[46]. Similarly, some non-conventional yeast strains markedly vary in aroma profile (i.e. concentrations of 2-phenylacetaldehyde and 2-phenylethan-1-ol) compared to the *S. cerevisiae* strain traditionally used in bakery[47].

Our findings illustrate how strain selection might be used to identify truffle strains with more desirable aroma and flavour. This is specially promising since *T. borchii* is the first species that has been successfully used to mycorrhize seedlings with axenic mycelial cultures instead of ascospores[48]. Overall, our results might also lead to the production of better quality truffle flavour and a higher consumer acceptance of truffle-flavoured food products (i.e. truffle-flavoured olive oil) that currently predominantly contain synthetic flavours.

Materials and Methods

Biological material. A total of 9 strains (axenic cultures) of *T. borchii* and one fruiting body (FB) were used in this study as described in Table 1.

For preparation of liquid cultures, *T. borchii* mycelia were pre-grown on malt extract (ME) agar (pH 7.0) [formula used per liter: malt extract 10 g (Difco & Becton Dickinson, Heidelber, Germany) and agar 15 g (Carl Roth, Karlsruhe, Germany)] for two months as described by Splivallo *et al.*[21], and half a colony transferred to 100 ml Erlenmeyer flasks containing 30 ml of ME broth (1%, pH 7.0). Liquid cultures were homogenized with a sterile grinder at 17,000 rpm (T 18 digital ULTRA-TURRAX, IKA, Staufen, Germany) for 10 s, and incubated at 23 °C for two months. Sample handling for subsequent experiments is illustrated in Fig. 1. along with the number of replicates used for each experiment.

Comparing the volatile profiles of different *T. borchii* mycelial cultures. Two-month-old mycelial cultures and malt extract broth without mycelium (negative controls) were homogenized at 17,000 rpm as described above, an aliquot of 5 ml in a 50 ml tube was subsequently pelleted by centrifugation (12,000 g for 10 min at 4 °C). The supernatant was discarded and a biomass of 200 ± 0.002 mg (wet weight) was transferred to a 20 ml SPME vial sealed with a screw cap and a silicon/polytetrafluoroethylene (PTFE) septum (VWR, Germany). Medium control samples were prepared by transferring 100 µl of malt extract to SPME vials. One fruiting body sample (originally frozen, but allowed to equilibrate to room temperature for 2 h before analysis) was also included for volatile analysis (300 ± 0.002 mg per SPME vial).

Volatiles extraction was performed by SPME in an autosampler (PAL RSI 85, CTC Analytics AG, Switzerland). Samples were first preheated to 60 °C for 20 min prior to extraction. Extraction was then done with an SPME fibre (PDMS/DVB/CARB Agilent Technologies, Waldbronn, Germany) exposed for 15 min in the headspace of the sample. Empty SPME vials were regularly inserted between samples to make sure no carry-over occurred. Volatiles were profiled by GC/MS (Agilent 7890B GC system equipped with a 5977B quadrupole MS detector (Agilent Technologies, Waldbronn, Germany). The SPME fibre was desorbed in the GC inlet (250 °C) in splitless mode and volatiles were separated on a capillary column (HP-5MS Agilent 19091S-433UI 0.25 mm × 30 m × 0.25 µm) by using the following program: start at 40 °C and hold for 5 min, ramp at a rate of 3 °C min^{-1} to 160 °C, ramp at a rate of 50 °C min^{-1} to 260 °C, hold for 1 min (total run time: 53 min). The carrier gas was helium with a flow rate of 1.2 ml min^{-1}. MS parameters were adjusted to a mass-to-charge ratio (m/z) scan range from 50 to 350, in an etune mode, ion M^+, with electron energy of 70 eV (MS source 230 °C, MS quad 150 °C).

Chromatograms were visualized with the Agilent Mass Hunter Qualitative Analysis software (version: B.07.00). To eliminate the shift in retention time due to the machine drifts, peaks were aligned using Tagfinder software version 4.1[28]. The intensity threshold was set to 3,000 but other parameters were the same as previously described in Sherif *et al.*[49]. As an output, Tagfinder created a matrix with sample names in columns, and TAGs (specific m/z values within a specific retention time window) in rows. Background noise (three times the values of the medium control samples (ME without mycelium)), was subtracted from all samples and TAGs for each sample were normalized by dividing their intensity by the total ion current (TIC) (Supplementary Table S1). Based on Supplementary Table S1, principal component analysis (PCA) was produced using the Past software version 3.04[50]. To create the heat map in Fig. 2B from Supplementary Table S1, a non-parametric test in R (version 3.2.3)[29] was used to identify TAGs that significantly differed among samples ($p < 0.05$, Kruskal-Wallis with $\alpha = 0.05$). Moreover, to avoid a biased representation in the heatmap towards tags with the highest intensities, TAG in single rows were divided by their maximum TAG intensity in that row. This resulted in relative concentrations between 0 and 1.

Identification of volatile compounds. Volatiles were identified by comparison of their mass fragmentation patterns with mass spectra databases (National Institute of Standards and Technology (NIST) library v. 2.0, Gaithersburg, USA), and by comparison of Kovats retention indices (calculated from n-alkanes) to literature values (http://www.pherobase.com/database/kovats/kovats-index.php, http://webbook.nist.gov/chemistry/gc-ri/). Moreover, authentic standards were used to confirm the identity of the following volatiles by GC/MS: 2-methylpropan-1-ol, 2-methylbutan-1-ol, 3-methylbutan-1-ol, oct-1-en-3-ol, 2-methylbutanal, 3-methylbutanal, 2-phenylethan-1-ol, benzaldehyde, 2-phenylacetaldehyde, DMDS, DMTS.

Confirming the Existence of the Ehrlich Pathway in *T. borchii* with ^{13}C labelled amino acids. Supplementation experiments with ^{13}C isotope labelled amino acids were performed with three mycelial strains (2, 3 and 5 in Table 1). Mycelial strains pre-grown in 30 ml malt extract liquid cultures were homogenised with

a grinder and washed and pelleted three times with 5 ml minimal medium. Minimal medium contained the following components (concentrations are given in mg/l): $MgSO_4.7H_2O$: 731, KNO_3: 80, KCl: 65, KH_2PO_4: 5, $MnCl_2.4H_2O$: 6, $ZnSO_4.7H_2O$: 4.52, H_3BO_3: 1.5, $CUSO_4$. $5H_2O$: 0.26, $(NH_4)_6Mo_7O_{24}.4H_2O$: 0.0046, $Ca(NO_3)_2.4H_2O$: 288, NaFeEDTA: 8, glycine: 3, thiamine: 0.1, pyrodoxine: 0.1, nicotinic acid: 0.5, myo-inositol: 50, KI: 0.75, sucrose: 10,000. After washing, mycelial pellets were dissolved in 5 ml minimal medium and 1.5 ml of this culture was transferred to an SPME vial. Vials were supplemented with 0.5 ml of an aqueous solution (sterile filtered) containing ^{13}C labelled L-methionine, L-leucine, L-isoleucine and L-phenylalanine (labels are shown in Fig. 3, final concentration of 5 mM in SPME vials) (Cambridge isotope laboratories, Andover, USA). Samples were incubated at room temperature on a 100 rpm orbital shaker (Neolab, Heidelberg, Germany) for 24 h in the dark. After incubation, mycelial volatiles were profiled by pressure-balanced headspace-GC/MS as described hereafter.

Volatiles were profiled using a Clarus 680 GC coupled to quadrupole Clarus SQ 8 C MS detector (PerkinElmer, MA, USA) equipped with a pressure-balanced headspace- autosampler (Headspace Sampler TurboMatrix 40, Perkin Elmer, MA, USA). Volatiles were extracted by pressure-balanced headspace in trap mode using the following program: 20 ml SPME vials sealed with a septum were pre-heated for 20 min at 70 °C (to increase the volatility of the analytes), then the vials were pressurized with helium (to 105 kPa for 2 min). 4-Bromofluorobenzene (100 µl of a 27.2 ppm IS in N2 (mol/mol)) was used as an internal standard (IS). The IS gas was injected into the vials prior to volatile adsorption by the trap (TurboMatrix air monitoring trap, Perkin Elmer) (loop load: 0.5 min, loop equilibration: 0.4 min, inject time 0.5 min). Volatiles were thermally desorbed from the trap and injected in the GC by increasing the temperature from 30 °C to 280 °C. Separation was performed on a capillary column Elite-5MS (30 m × 0.25 mm i.d., 1.00 µm film thickness, Perkin Elmer). The GC oven temperature program was: start at 50 °C and hold for 1 min, 15 °C min^{-1} to 180 °C and hold for 1 min, 100 °C min^{-1} to 300 °C and hold for 5 min (total run time: 16.87 min). The carrier gas was helium with constant pressure at 75 kPa. MS operated electron impact ionisation (EI) conditions (70 eV) with scan range from m/z 50-300 (MS source 200 °C). Turbo Mass software (version: 6.1.0, PerkinElmer) was used to visualize the chromatograms and the mass spectrums. Mass spectra of mycelial strains supplemented with ^{13}C labelled amino acids was compared to the mass spectra of non-labelled amino acids supplemented mycelia to inspect whether the labels were integrated into the targeted volatiles (see Supplementary Fig. S1).

Testing the induction of Ehrlich volatiles in *T. borchii*. The supplementation experiment with non-labelled amino acids was carried out using all the nine mycelial strains of Table 1. Mycelial cultures and controls with no mycelium in ME broth were homogenized with a sterile grinder and were divided into aliquots of 2 ml in SPME vials. Vials were supplemented with 0.4 ml of an aqueous solution containing non-labelled methionine, leucine, isoleucine and phenylalanine (sterile filtered, with a final concentration of 20 mM in SPME vials) (Carl Roth, Karlsruhe, Germany) or water (control). Samples were kept at room temperature on a 100 rpm orbital shaker for 24 h in the dark. Mycelial volatiles were profiled as for the samples containing labelled amino acids. To determine the mycelial biomass, 3 ml of each homogenized culture was transferred to 50 ml Falcon tube, the mycelium was separated by centrifugation from its supernatant and wet weight was recorded. Peak areas (PA) of target volatiles were subsequently normalized to the peak area of the internal standard and to the biomass of each sample (Fig. 5).

Sensory tests with *T. borchii* mycelial strains. Three sensory tests were performed in total, aimed at various objectives. A first test was done to evaluate if the human nose perceived induced volatiles in *T. borchii* strains supplemented with methionine (comparison: strain 2 either unsupplemented (water control)) or supplemented with 5 mM methionine. The second test was performed to investigate whether the human nose could differentiate between the aromas induced by methionine (strain 2, 5 mM) or a mixture of amino acids (strain 2 and leucine, isoleucine, phenylalanine, and methionine, each 5 mM) in the same strain. A third test assessed the ability of the human nose to distinguish between strains (strains 3 and 9, both supplemented with 5 mM phenylalanine).

For all the sensory tests, panellist (14 individuals, 6 women and 8 men) were presented with a questionnaire comprising two parts: a discriminatory test ("triangle test" as described in O'Mahony *et al.*[51]) and assessment of aroma attributes. For the triangle test, each panellist was presented with three samples (two identical and one "different") and asked to identify the two samples judged the most similar by their smells. Results of the triangle test were analysed using a one-sided binomial proportions test[52]. For the assessment of aroma attributes, each panellist was presented with four samples (two replicates of each sample). They were asked to indicate the presence of the following aroma attributes; sulfur/garlic, mushroom, floral, straw/malt, fermented/roasted, followed by rating the "overall aroma intensity" using a four-point category scale (0 indicating 'no smell' and 3 'very intense smell'). These aroma attributes were provided to the assessors as representative aroma attributes typically used to describe the volatiles of Fig. 5. For each aroma descriptor, frequencies were expressed as a percentage of total possible counts (14 panellist × 2 replicates = 28 counts), yielding values between zero and one. Volatile profiles from the samples used for sensory analysis were generated by pressure-balanced headspace -GC/MS as previously described.

Ethics statement. Use of human subjects for this study was reviewed by the Ethics Committee of the medical department of the University of Frankfurt and was granted exempt status. Informed consent was obtained from all participants.

Data Availability. The data generated or analysed during this study are included in this published article (and its Supplementary Information files).

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Author Contributions
R.S. and M.V. conceived the experiments, M.V. conducted the experiments. R.S. and M.V. analysed the results. All authors reviewed the manuscript.

Additional Information
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Competing Interests: M.V. declares no competing interests in relation to the work described. As financial interest, R.S. has filed a patent that deals with the production of truffle flavour from truffle mycelium. R.S. declares no further competing interests in relation to the work described.

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Supplementary Figure

Improving truffle mycelium flavour through strain selection targeting volatiles of the Ehrlich pathway

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Fig. S1 – Part 1

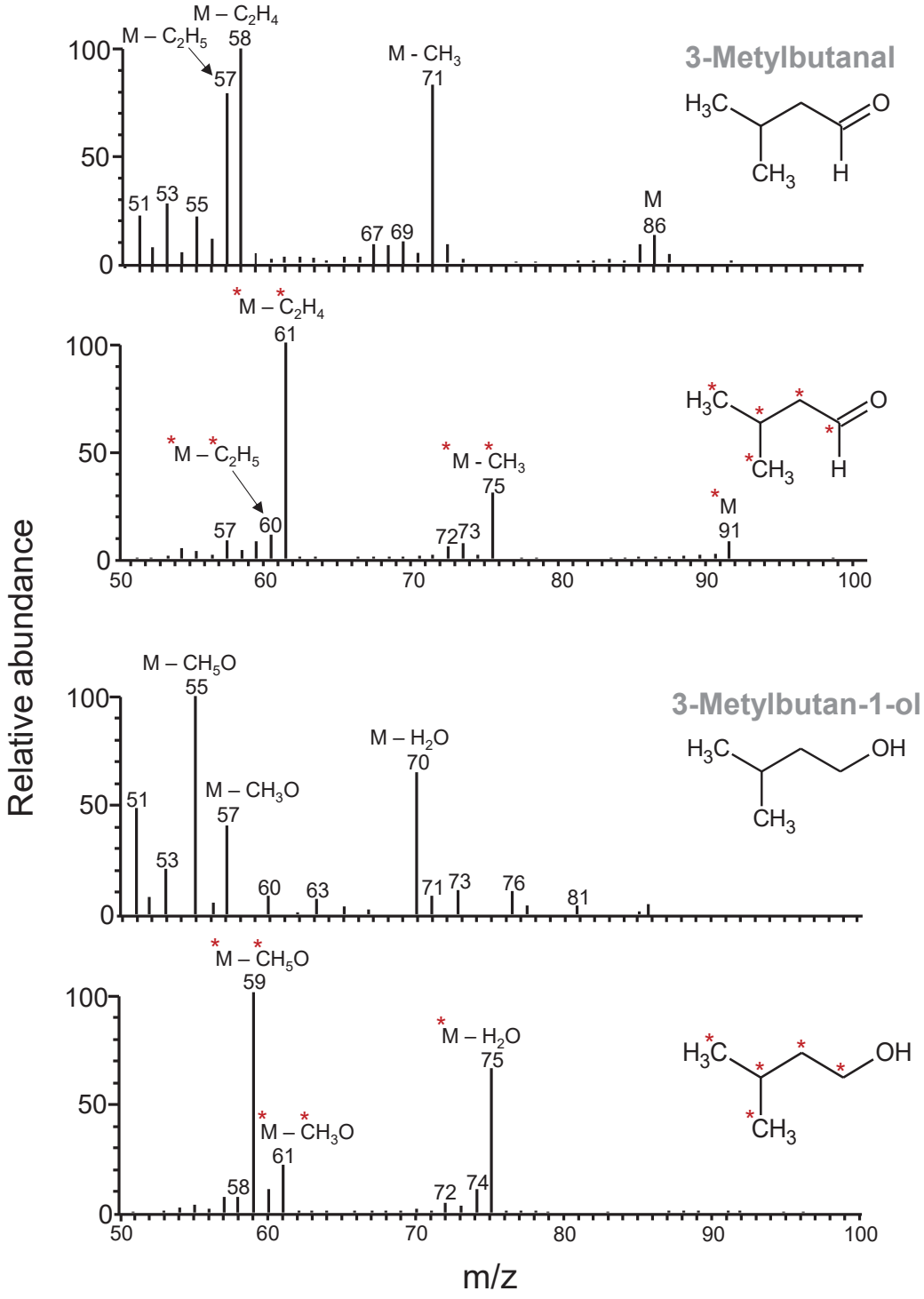
3-Metylbutanal
M – C2H4
M – C2H5
58
57
M - CH3
71
51
53
55
67
69
M
86
*M – *C2H4
61
*M – *C2H5
*M - *CH3
75
57
60
72
73
*M
91
3-Metylbutan-1-ol
M – CH5O
55
M – H2O
70
M – CH3O
57
51
53
60
63
71
73
76
81
*M – *CH5O
59
*M – H2O
75
*M – *CH3O
61
58
72
74
Relative abundance
m/z

Fig. S1 – Part 2

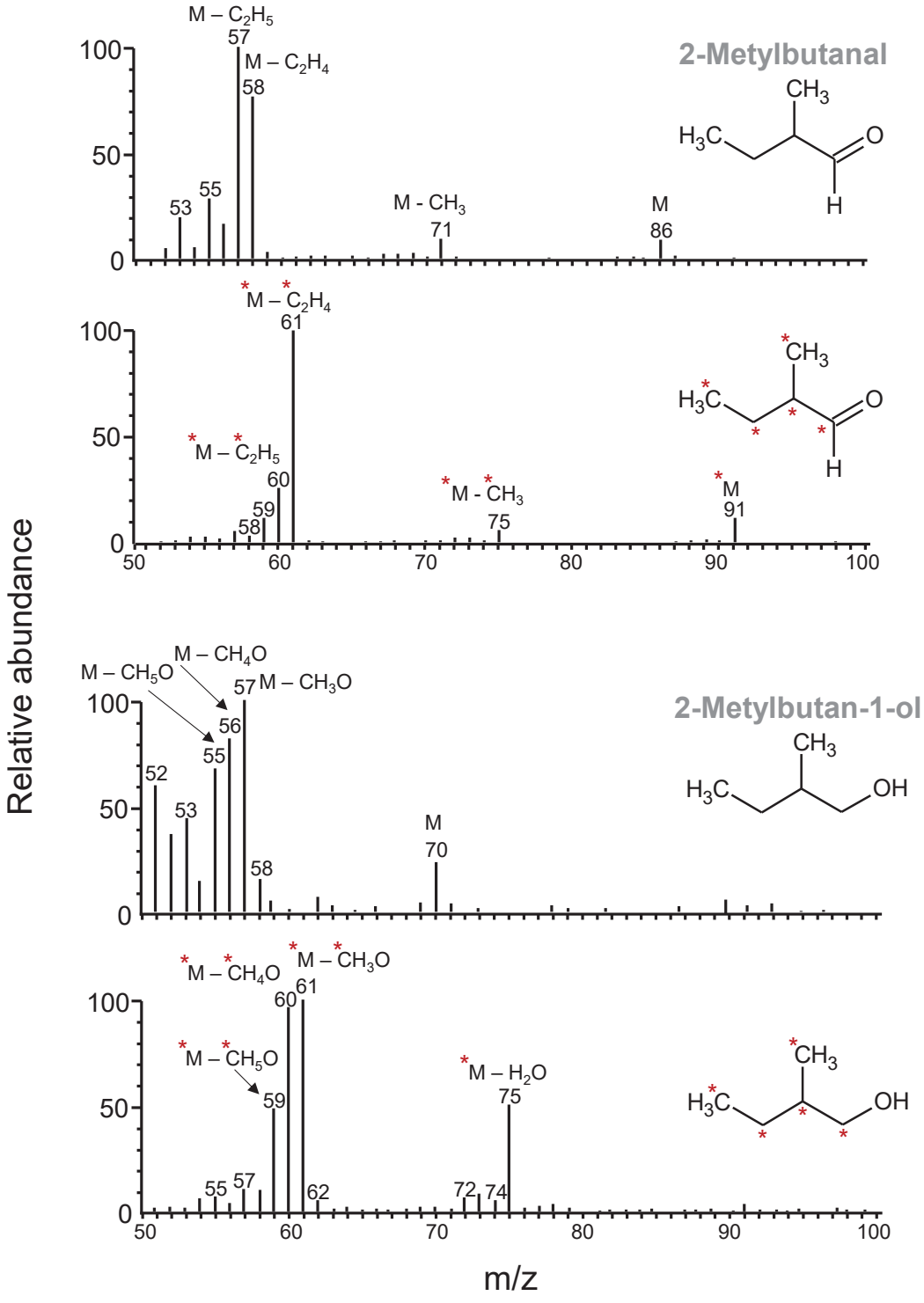
2-Metylbutanal
M – C2H5
57
M – C2H4
58
M - CH3
71
M
86
53
55
100
50
0
*M – *C2H4
61
*M – *C2H5
60
59
58
*M - CH3
75
*M
91
50
60
70
80
90
100
2-Metylbutan-1-ol
M – CH5O
M – CH4O
57
M – CH3O
56
55
52
53
58
M
70
*M – *CH4O
*M – *CH3O
60
61
*M – *CH5O
59
*M – H2O
75
55
57
62
72
74
Relative abundance
m/z

Fig. S1 – Part 3

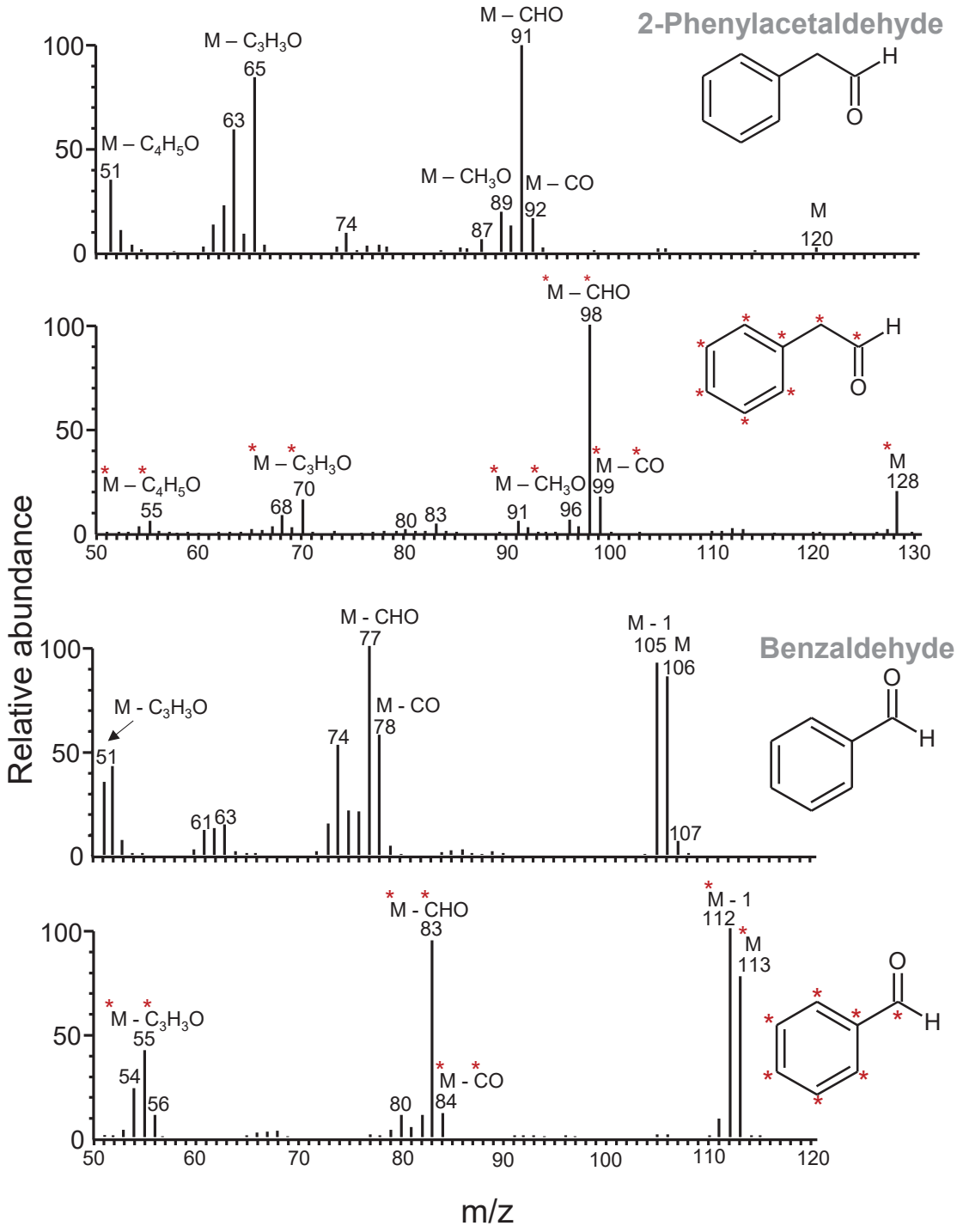
2-Phenylacetaldehyde
M – CHO
91
M – C3H3O
65
63
M – C4H5O
51
M – CH3O
89
M – CO
92
74
87
M
120
*M – *CHO
98
*M – C4H5O
55
*M – *C3H3O
70
68
80
83
*M – CH3O
91
96
*M – CO
99
*M
128
Benzaldehyde
M - CHO
77
M - 1
105 M
106
M - C3H3O
51
M - CO
78
74
61
63
107
*M - CHO
83
*M - 1
112
*M
113
*M - *C3H3O
55
54
56
*M - CO
80
84
Relative abundance
m/z

Fig. S1 – Part 4

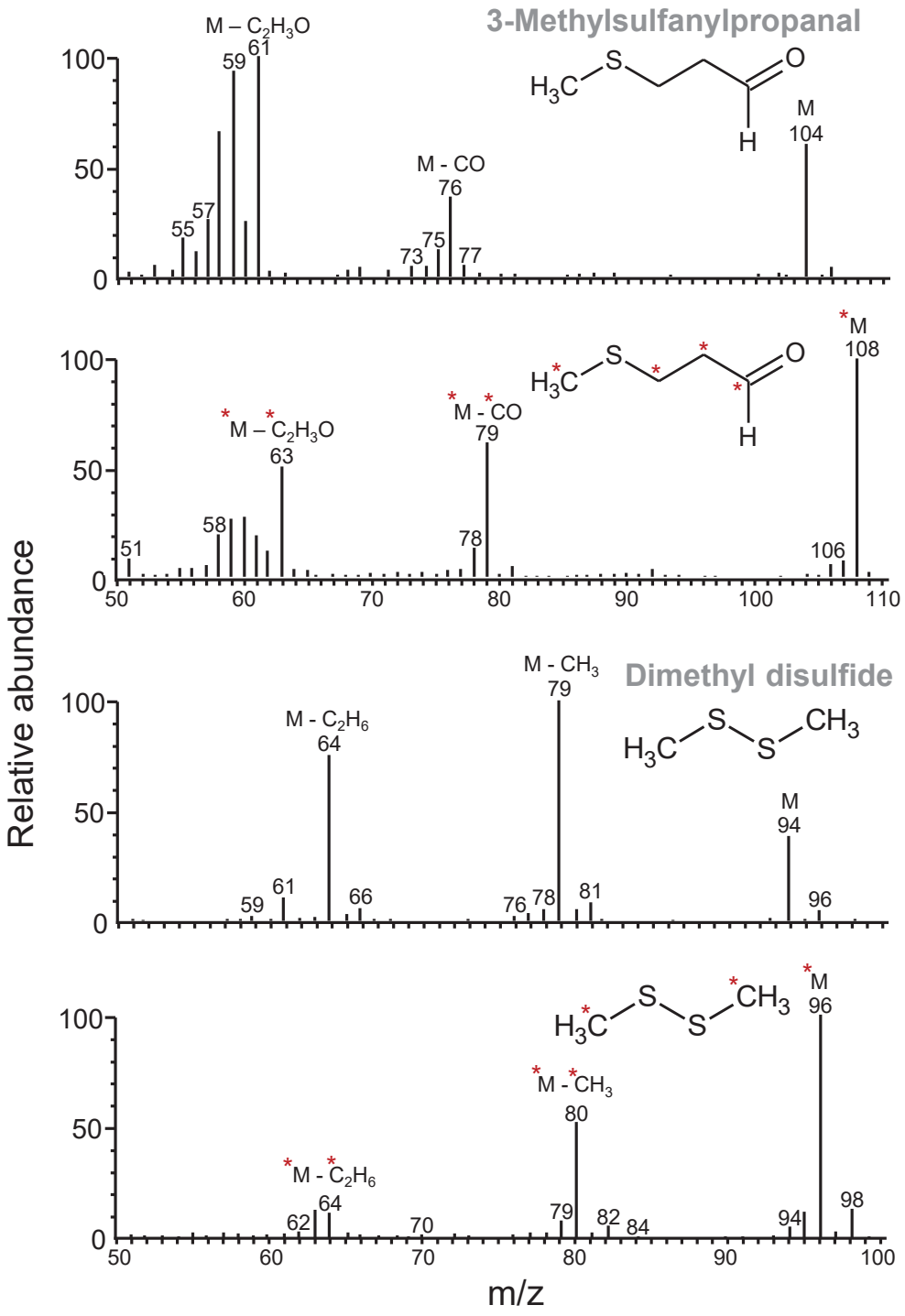
3-Methylsulfanylpropanal
M – C2H3O
M - CO
M
104
*M
108
*M – *C2H3O
*M - *CO
Dimethyl disulfide
M - CH3
M - C2H6
M
94
*M
96
*M - *CH3
*M - *C2H6
Relative abundance
m/z

Fig. S1 – Part 5

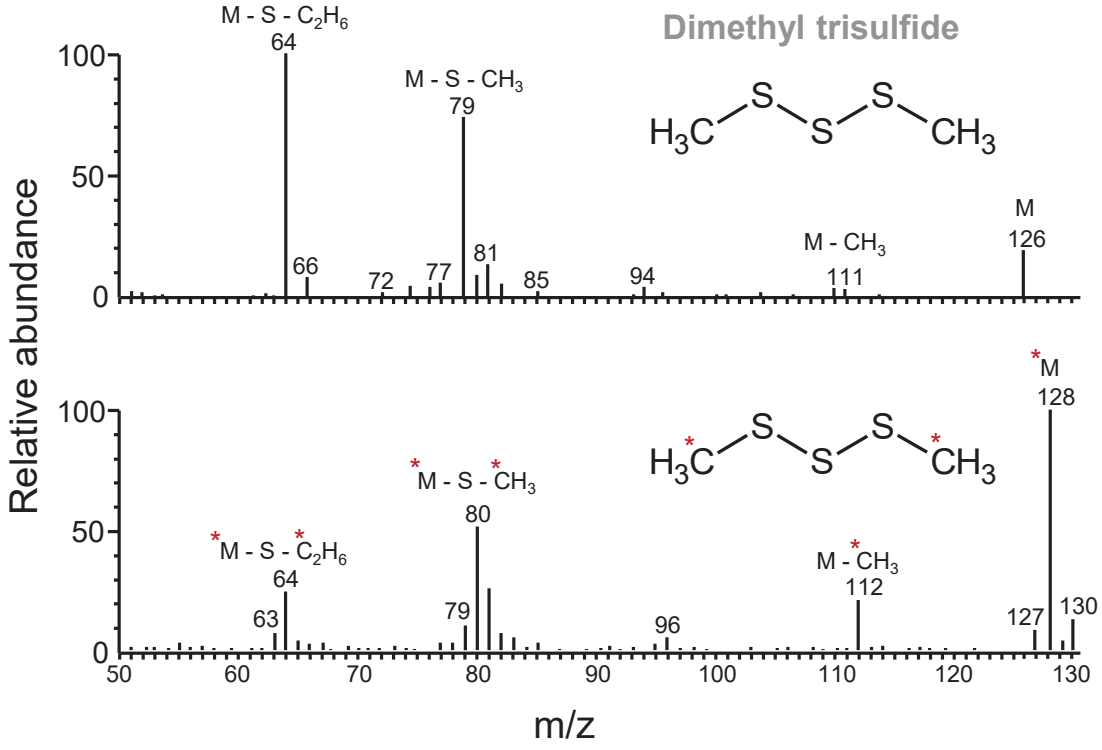

Figure S1. Demonstrating the existence of the Ehrlich pathway in *T. borchii*.

Mass spectra of volatiles derived from the Ehrlich pathway upon supplementing mycelial cultures with unlabelled and ^{13}C labelled (*) amino acids (leucine, isoleucine, phenylalanine, and methionine at 5 mM). Mass spectra illustrate the full incorporation of the labelled carbon atoms into target volatiles. Results for strains 2, 3 and 5 were equivalent.

7.2 Chapter 2: The role of the microbiome of truffles in aroma formation: a meta-analysis approach

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Contributing authors: Maryam Vahdatzadeh (MV), Aurelie Deveau (AD), Richard Splivallo (RS)

Contributions of doctoral candidate and co-authors

(1) Concept and design

MV: 50%; RS: 50%

(2) Conducting tests and experiments

n.a.

(2) Literature collection

MV: 60%; AD: 10%; RS: 30%

(3) Compilation of data sets and figures

MV: 80%; RS: 20%

(4) Analysis and interpretation of data

MV: 40%; AD: 20%; RS: 40%

(5) Drafting of manuscript

MV: 40%; AD: 20%; RS: 40%

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MINIREVIEW

The Role of the Microbiome of Truffles in Aroma Formation: a Meta-Analysis Approach

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Truffles (*Tuber* spp.) are ascomycete subterraneous fungi that form ectomycorrhizas in a symbiotic relationship with plant roots. Their fruiting bodies are appreciated for their distinctive aroma, which might be partially derived from microbes. Indeed, truffle fruiting bodies are colonized by a diverse microbial community made up of bacteria, yeasts, guest filamentous fungi, and viruses. The aim of this minireview is two-fold. First, the current knowledge on the microbial community composition of truffles has been synthesized to highlight similarities and differences among four truffle (*Tuber*) species (*T. magnatum*, *T. melanosporum*, *T. aestivum*, and *T. borchii*) at various stages of their life cycle. Second, the potential role of the microbiome in truffle aroma formation has been addressed for the same four species. Our results suggest that on one hand, odorants, which are common to many truffle species, might be of mixed truffle and microbial origin, while on the other hand, less common odorants might be derived from microbes only. They also highlight that bacteria, the dominant group in the microbiome of the truffle, might also be the most important contributors to truffle aroma not only in *T. borchii*, as already demonstrated, but also in *T. magnatum*, *T. aestivum*, and *T. melanosporum*.

Microbes can be found almost everywhere on our planet. They colonize many different types of habitats, among them living organisms, such as plant roots or insect and human guts. Classical microbiological methods have long offered a spotlight view on microbial diversity. Recent high-throughput molecular techniques have revolutionized the field of microbial ecology by unraveling an enormous microbial diversity in numerous organisms and highlighting the deep impact of microbiomes of their host physiology and behavior (1, 2). Truffle fungi are no exception, since they are colonized by a complex microbial community made up of bacteria, yeasts, guest filamentous fungi, and viruses (3–14).

Truffles are subterranean ascomycete fungi that form ectomycorrhizas in symbiotic relationship with plant roots (15). Their fruiting bodies are appreciated for their distinctive aroma, which is partially derived from microbes (6, 14, 16). The aim of this minireview is to synthesize the current knowledge on the composition of the microbial community of truffles and discuss their potential role in truffle aroma formation, specifically focusing on volatiles that are responsible for human-perceived truffle aroma (defined as odorants).

TRUFFLE MICROBIOMES

Truffles are colonized by microbes at all stages of their life cycle, which include a symbiotic stage in association with a host plant (ectomycorrhiza), a sexual stage (fruiting bodies), and a free-living mycelial stage, which might serve an exploratory purpose in the soil. To date, microbes and microbial communities have been characterized in truffles with culture-dependent and -independent techniques in >15 papers (3–14, 17–21). Various life cycle stages of four commercially relevant *Tuber* species have been investigated: the white truffles *T. magnatum* and *T. borchii* and the black species *T. melanosporum* and *T. aestivum*. Similarities and differences in the compositions of the microbial community of truffle species are highlighted here for bacteria, fungi (yeast and filamentous), and viruses.

BACTERIAL COMMUNITIES

Most studies investigating microbes in truffles have been performed on bacteria. These bacteria can heavily colonize the inner and outer parts of truffle fruiting bodies, as their densities range from a million to a billion cells per gram (dry weight) of fruiting bodies (4, 5, 19–22). The aims of these studies ranged from the characterization of taxonomic and/or functional community composition to the influence of specific variables. Indeed, bacterial community composition has been investigated in relation to fruiting body maturation, aging, season or life cycle (i.e., mycorrhizas versus fruiting body), and tissue specificity (the gleba [the inner part of the fruiting bodies] versus the peridium [the outer protective layer]).

Combinations of culture-dependent and -independent methods have demonstrated that all truffle species analyzed so far are colonized by complex bacterial communities made mostly of *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* (Fig. 1) (4, 5, 12–14, 19). Similarities among fruiting bodies of the three truffle species investigated to date include a dominance of *Alphaproteobacteria* and a relative paucity of *Firmicutes* and *Actinobacteria*. On the contrary, differences among truffle species might exist for *Betaproteobacteria*, *Gammaproteobacteria*, and *Bacteroidetes*, which might be more abundant in *T. borchii* than in *T. melanosporum* and *T. magnatum* (Fig. 1). As a matter of fact, a *Bacteroidetes* strain might even coexist inside *T. borchii* mycelia

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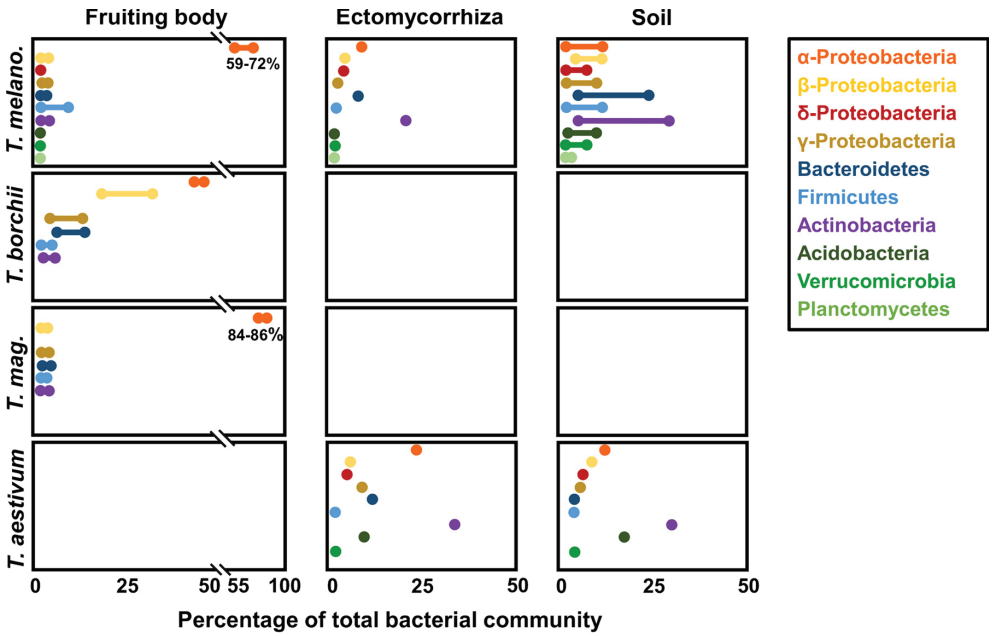

FIG 1 Bacterial communities in fruiting bodies, ectomycorrhizas, and soil. The most abundant bacterial communities associated with four truffle species based on culture-independent methods are shown. The bars represent the minimum and maximum values reported in the literature, whereas the points display a single literature value (*T. aestivum*, reference 12; *T. magnatum* [*T. mag.*], reference 5; *T. borchii*, references 4 and 14; and *T. melanosporum* [*T. melano.*], reference 24 [and for the period from December to January, reference 13 for the gleba). Cells for which no literature data were available were left empty.

grown under axenic laboratory conditions (18), suggesting a possible tight association between bacteria and truffles. The occurrence of endosymbionts has not been described so far in other truffle species.

The bacterial community composition of truffle fruiting bodies might evolve over time and in relation to the physiology of the truffle host. Indeed, truffle fruiting bodies mature as their inner part (gleba) undergoes melanization due to the spore-forming process taking place inside the fungal asci. This maturation/melanization process generally lasts a few months and occurs in late autumn/winter for *T. borchii*, *T. melanosporum*, and *T. magnatum* harvested in Europe. Using fluorescence *in situ* hybridization (FISH) in the latter species, a slight but significant decrease in total bacterial count was observed with increasing maturity; nevertheless, no difference in the relative community composition was detectable for *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Bacteroidetes*, *Firmicutes*, or *Actinobacteria* (5). A different pattern was observed in *T. melanosporum* using high-throughput sequencing methods (13). The composition of the bacterial community present inside the gleba and in the peridium significantly changed along the course of the maturation of the ascocarps. The community composition in the peridium was very close to that of the soil community in young ascocarps but strongly diverged from the soil community in mature ascocarps. The differences were mainly in the peridium, due to a progressive increase in the abundance of *Bacteroidetes* and *Alphaproteobacteria*, while the abundance of *Betaproteobacteria* members decreased. In contrast, the glebal bacterial community was dominated very early by *Alphaproteobacteria*. Moreover, this dominance kept increasing with the maturity level, just as it did in the peridium. All together, these data prompted Antony-Babu et al. (13) to propose the following model: soil bacteria would colonize truffle primordia before the differentiation of ascocarpic tissues would occur. Next, the bacteria would be trapped in the gleba and partly protected from soil exchanges by the warted peridium. Because of this compartmentalization, bacterial community composition would mainly evolve in response to changes in the physiology of the maturing ascocarp. In contrast, the peridium would remain in contact with the soil all along the development process of the ascocarp, due to cracks that open during growth of the ascocarp (13).

In addition to natural variations, the harvest of truffle fruiting bodies is likely to induce changes in the composition of the associated bacterial community. This might be due to modifications in physicochemical parameters, such as temperature and CO_2 level (23). For example, Splivallo et al. (14) observed the appearance of colonies belonging to *Firmicutes* and *Actinobacteria*, while the abundance of members of *Alphaproteobacteria* and *Betaproteobacteria* decreased in fruiting bodies of *T. borchii* after 6 days of post-harvest storage at room temperature (14).

The composition of bacterial communities associated with truffles is influenced not only by the stage of maturity of the fruiting bodies but also by the stage of the life cycle of the fungus. Comparative analysis of the bacterial communities associated with fruiting bodies and ectomycorrhizae (EcM) of *T. melanosporum* showed striking differences, suggesting that the fungus might provide two different habitats to bacteria. For example, *Actinobacteria* are dominant in EcM but rare in fruiting bodies of *T. mela-*

nosporum (13). Interestingly, enrichment in several genera of *Actinobacteria* has also been demonstrated for specific zones within orchards of *T. melanosporum*, referred to as brûlés (24), which are especially rich in truffle mycelia (25).

Overall, these observations demonstrate that truffles provide several habitats to complex bacterial communities. Among the *Alphaproteobacteria*, members of the *Bradyrhizobiaceae* and *Rhizobiaceae* families mainly seem to form the core component of these communities, whatever the truffle species considered. The parameters that control the selection of this very specific community are still to be discovered. A tempting hypothesis is that truffle fruiting bodies would be more than a habitat for bacteria and that mutualistic interactions might occur between the fungi and their microbiota. Some members of the *Rhizobiales* order are well known for their ability to fix atmospheric nitrogen either as free-living organisms or in symbiosis with plants (26). Barbieri et al. (27) demonstrated that nitrogen fixation occurs inside fruiting bodies of the white truffle *T. magnatum*. Nif genes encoding the enzymes responsible for nitrogen fixation were also detected in bacteria associated with *T. melanosporum* (13). Thus, it is tempting to speculate that part of the nitrogen captured by bacteria in fruiting bodies might benefit the host fungus. However, it remains to be demonstrated that the nitrogen fixed by bacteria inside truffle fruiting bodies is indeed transferred to the fungus.

YEAST COMMUNITIES

Besides bacteria, yeasts are ubiquitous organisms that occupy most terrestrial ecological niches. Yeast community composition has been investigated in fruiting bodies (*T. aestivum*, *T. melanosporum*, and *T. magnatum*), ectomycorrhizas, and truffle orchard soil (*T. aestivum*) (3, 6, 21). These studies were based on culture-dependent methods and might hence miss the real diversity that exists; nevertheless, they also do provide useful insights. In a study comparing yeast distribution within an orchard of *T. aestivum*, Zacchi et al. (3) demonstrated that yeasts were enriched on truffle ectomycorrhizas and fruiting bodies, reaching up to 3×10^7 CFU/g of fruiting bodies (dry weight) compared to that in bulk soil (1×10^2 CFU/g of fruiting bodies [dry weight]). The total yeast diversity was made of five species, namely, *Cryptococcus albidus*, *Cryptococcus humicola*, *Rhodotorula mucilaginosa*, *Debaryomyces hansenii*, and *Saccharomyces paradoxus* (3). Interestingly, *Cryptococcus* spp., *R. mucilaginosa*, *D. hansenii*, and *Saccharomyces* spp. were also isolated by others (6, 21) from *T. melanosporum*, *T. magnatum*, or *T. aestivum* and might therefore be common to distinct truffle species. Yeast density might also vary between the peridium and gleba. Indeed, based on culture-dependent methods, yeasts were isolated only from the peridium of *T. aestivum* and *T. melanosporum* (10^3 to 10^4 CFU/g of fruiting bodies [fresh weight]) but not from the gleba of intact truffles (21).

These observations suggest that, as with bacteria, yeast community composition might vary with tissues, and a "core yeast community" might exist among truffle species. Culture-independent techniques will nevertheless be necessary to confirm these hypotheses and get a better view of the variability in space and time of the yeast communities associated with truffles.

GUEST FILAMENTOUS FUNGI AND VIRUSES IN TRUFFLES

Besides being colonized by yeasts and bacteria, truffles may also be colonized by filamentous fungi (guest filamentous fungi as opposed to the host truffle mycelia) and viruses. As in the case of yeasts, only a few reports exist on the occurrence of viruses in truffles. Guest filamentous fungi, mostly ascomycetes, have been isolated from the *Tuber* species *T. rufum*, *T. brumale*, *T. magnatum*, *T. melanosporum*, *T. nitidum*, *T. excavatum*, *T. aestivum*, *T. borchii*, and *T. puberulum* (7). However, their occurrence in fruiting bodies might be seldom, since guest filamentous fungi were isolated from only 26% of all truffles ($n = 30$), suggesting a loose association. The density of guest filamentous fungi might vary between the gleba and peridium. In *T. melanosporum* and *T. aestivum*, corresponding with what has been observed for yeasts, guest filamentous fungi (ascomycete molds) predominantly colonized the peridium, with a density of 10^2 CFU/g of fruiting bodies (fresh weight), but they seem to be absent from the gleba (21). Similarly, a recent report described the occurrence of viruses (*Totivirus*, *Mitovirus*, and *Endornavirus* from *T. aestivum* and *Mitovirus* from *T. excavatum*) without, however, addressing their frequency of occurrence within fruiting bodies or in orchards (8–11). Some authors have also suggested viral gene integration in the genome of *T. melanosporum* (28). Surely, guest filamentous fungi and viruses might interact with truffles in nature; however, additional ecological data are needed at this stage to understand how frequently they might occur and to assess how relevant they are in the microbiome of truffles.

INVOLVEMENT OF MICROBES IN PRODUCTION OF AROMA THAT HUMANS PERCEIVE AS THE TRUFFLE SMELL

Unique and delightful aromas are partially responsible for the high demand of truffles in the world market. The particular aromas of truffles are made up of a mixture of various volatiles, namely alcohols, esters, ketones, aldehydes, and aromatic and sulfur compounds. To date, the number of identified volatiles from various truffle species is >200; however, only a small fraction of these, the so-called odorants, are responsible for what humans perceive as the truffle smell (16, 29, 30).

Historically, the aroma of the white truffle *T. magnatum* was the first one characterized and ascribed to a single sulfur compound (2,4-dithiapentane) (31). In the 1980s, a mixture of two constituents, 2-methylbutanal and dimethyl sulfide, were patented to reproduce the smell of the Périgord truffle *T. melanosporum* (32). Essentially, due to increasingly sensitive techniques in sensory science, the number of key odorants in *T. melanosporum* was recently revised to >15 volatiles (29). A comparable number of odorants (about 10 to 20) have also been described in four black truffle *Tuber* species (*T. aestivum*, *T. himalayense*, *T. indicum*, and *T. sinense* [29, 30]) and in the white truffle *T. borchii* (16). Interestingly, most of these odorants are common to almost all truffle species (i.e., methylthiomethane, 3-methyl-1-butanol, and oct-1-en-3-ol), and only a few are species specific or occur in a rather small number of species (i.e., thiophene derivatives and 2,4-dithiapentane) (Fig. 2). The exact origin of truffle volatiles and specifically of most odorants reported in Fig. 2 is unclear. It has been speculated that truffle aroma might result from the intimate interaction of truffles and their microbiomes (6, 33, 34). Indeed, some volatiles might be produced by both truffles and microbes, while others might be derived from a single player (i.e., yeasts, bacteria, or truffles). Only recently has the role of bacteria in the formation of thiophene derivatives, odorants unique to *T. borchii*, been demonstrated. In the latter species, only bacteria and not truffles metabolize a precursor of unknown origin into volatile thiophene derivatives (14). As the matter of fact, the biosynthetic

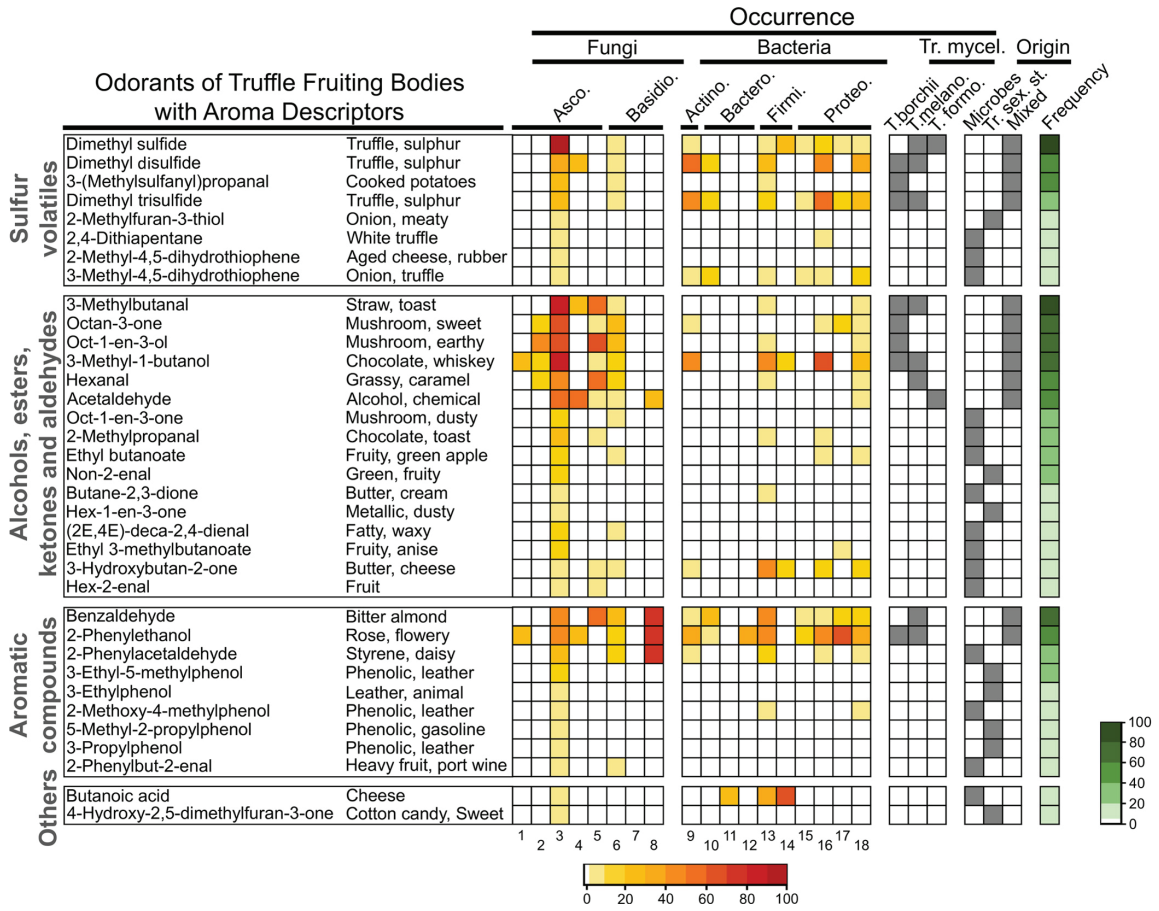

FIG 2 Ability of microbes to produce typical odorants of truffle fruiting bodies. List of odorants and aroma descriptors from *T. melanosporum* (*T. melano.*) and *T. aestivum* (29), *T. indicum*, *T. himalayense*, and *T. sinense* (30), *T. borchii* (16), and *T. magnatum* (31). Occurrences in fungal and bacterial phyla/classes are derived from the mVOC database (37) and the data from a review on fungal volatiles (38). They are shown as a heatmap representing the percent occurrence in each class, with *n* being the total number of organisms in each class (for *Ascomycetes* [*Asco.*]: 1, *Dothideomycetes* [*n* = 4]; 2, *Eurotiomycetes* [*n* = 29]; 3, *Pezizomycetes* [*n* = 26]; 4, *Saccharomycetes* [*n* = 4]; and 5, *Sordariomycetes* [*n* = 47]; for *Basidiomycetes* [*Basidio.*]: 6, *Agaricomycetes* [*n* = 135]; 7, *Exobasidiomycetes* [*n* = 3]; and 8, *Pucciniomycetes* [*n* = 4]; for *Actinomycetes* [*Actino.*]: 9, *Actinobacteria* [*n* = 62]; for *Bacteroidetes* [*Bactero.*]: 10, *Bacteroidetes* [*n* = 17]; 11, *Bacteroidia* [*n* = 24]; and 12, *Flavobacteria* and *Sphingobacteria* [*n* = 3]; for *Firmicutes* [*Firmi.*]: 13, *Bacilli* [*n* = 55]; and 14, *Clostridia* [*n* = 10]; for *Proteobacteria* [*Proteo.*]: 15, *Alphaproteobacteria* [*n* = 25]; 16, *Betaproteobacteria* [*n* = 43]; 17, *Deltaproteobacteria* [*n* = 16]; and 18, *Gammaproteobacteria* [*n* = 61]). Occurrence in axenic cultures of truffle (Tr. mycel.) is shown as the presence/absence for *T. borchii* (33, 40, 48), *T. melanosporum* (41), *T. formosanum* (*T. formo.*) (42). Origin refers to the speculative origin of the odorants in truffle fruiting bodies, where some odorants could be produced by microbes only (microbes), by truffle only at its sexual stage (Tr. sex. st.), or by both microbes and truffles (mixed). The frequency represents the percent occurrence of each odorant in fruiting bodies of 13 truffle species (*T. aestivum*, *T. brumale*, *T. himalayense*, *T. indicum*, *T. sinense*, *T. melanosporum*, *Tuber mesentericum*, *T. borchii*, *T. excavatum*, *T. magnatum*, *Tuber oligospermum*, *Tuber panniferum*, and *T. rufum* [16, 29–31, 37]).

pathway leading to thiophene derivatives remains elusive (14), and this is also the case for 2,4-dithiapentane, the major odorant of *T. magnatum*. In contrast, based on the genome of *T. melanosporum*, pathways leading to odorants commonly produced by yeasts and bacteria most likely exist in truffles as well (34, 35). This is the case, for example, for the Ehrlich pathway, which consists of the catabolism of specific amino acids and results in dimethyl sulfide, 2-phenylethanol, 2- and 3-methylbutanol, and numerous other volatiles common to microbes and truffles (35). The Ehrlich pathway consists of a three-step process involving the initial transamination of an amino acid, followed by decarboxylation and reduction steps (36). Indeed, enzymes fulfilling these steps most likely exist in *T. melanosporum* (34, 35); their functions have nevertheless not yet been demonstrated. At this stage, however, genomes provide limited insights on the possible identity of the producer of specific odorants, because either the pathways leading to those odorants are highly conserved among yeasts, bacteria, and truffles (i.e., the Ehrlich pathway) or the biosynthetic pathways are not known (i.e., thiophene derivatives and 2,4-dithiapentane).

By combining knowledge about the structure of truffle microbiomes (Fig. 1) with literature data on the ability of specific microbes to produce odorants, we speculate here on the origin of

these volatiles in truffles. For this purpose, we first established a list of all odorants described in truffles and reported in four publications (16, 29–31). We then used the mVOC database (37) and the data from a review on fungal volatiles (38) to understand which organisms had the ability to produce those volatiles, specifically focusing on the phyla and classes reported in Fig. 1. For the purpose of this review, volatile occurrence is expressed for bacterial and fungal phyla/classes and presented as a heatmap in Fig. 2.

SULFUR-CONTAINING VOLATILES

Sulfur-containing volatiles (sulfur volatiles) represent the most important group of odorants in truffles, since they confer the typical garlicky and sulfurous notes that characterize all truffle species (see the aroma descriptors in Fig. 2). The most common sulfur-containing volatile in truffle fruiting bodies is dimethyl sulfide, which has been detected in 85% of the species investigated to date (Fig. 2). Along with dimethyl disulfide, dimethyl trisulfide, and 3-(methylsulfanyl)propanal, dimethyl sulfide might be derived from the catabolism of methionine through the Ehrlich pathway (36, 39, 48). According to the mVOC database on microbial volatiles (37), the last four volatiles occur in the fungal classes of *Pezizomycetes* (i.e., truffles) and *Agaricomycetes* and in eight bacterial classes (Fig. 2). Since most of these volatiles are also produced by axenic cultures of truffle mycelia (40–42, 48), they might be synthesized in truffle fruiting bodies by both bacteria and truffle mycelia. Of special interest is dimethyl sulfide, since it might be produced by some *Alphaproteobacteria* and *Betaproteobacteria* (Fig. 2), which are also dominant in truffle fruiting bodies (Fig. 1).

In contrast to the relatively common sulfur volatiles just described, other sulfur odorants might be more specific (i.e., specific to a single or a limited number of species). Four sulfur volatiles, namely, 2-methyl-4,5-dihydrothiophene, 3-methyl-4,5-dihydrothiophene, 2,4-dithiapentane, and 2-methylfuran-3-thiol, occur in one or two truffle species only (Fig. 2). As for the common sulfur-containing volatiles, they might be derived from methionine; however, this has not yet been appropriately demonstrated (i.e., through feeding with labeled precursors). Interestingly, none of these specific sulfur odorants have been reported in axenic cultures of truffle mycelia, but some microbes have the ability to produce them (Fig. 2). Based on this observation, 2,4-dithiapentane might be produced by *Betaproteobacteria* in *T. magnatum* fruiting bodies. Interestingly, this might be a case similar to the one of thiophene derivatives, which were recently shown to originate from bacteria inhabiting *T. borchii* (14). 2-Methylfuran-3-thiol has been reported in fruiting bodies of *T. melanosporum* and *T. aestivum* (29), but this volatile has been not detected in either axenic mycelial cultures or microbes (Fig. 2). Its origin, therefore, remains elusive; nevertheless, it can be speculated that the latter odorant might be specifically produced during the sexual stage of truffles.

Overall, this suggests that common sulfur volatiles might be produced inside truffle fruiting bodies by both truffles and microbes (mixed origin), whereas more specific sulfur volatiles might be derived from truffles or microbes only.

ALCOHOLS, ESTERS, KETONES, AND ALDEHYDES

Another important group of truffle odorants is made of alcohols, esters, ketones, and aldehydes that are possibly derived from amino acid and fatty acid catabolism (36). As for sulfur volatiles, some commonly occur in numerous truffle species, while others are more specific (Fig. 2). Axenic cultures of truffle mycelia and numerous fungal and bacterial phyla are able to produce the most common volatiles (3-methylbutanal, octan-3-one, oct-1-en-3-ol, 3-methyl-1-butanol, hexanal, and acetaldehyde) which occur in >50% of all species. Interestingly enough, eight-carbon-containing volatiles (i.e., octan-3-one and oct-1-en-3-ol) were believed to be strictly of fungal origin, but Fig. 2 suggests that like 3-methylbutanal, 3-methyl-1-butanol, hexanal, and acetaldehyde, they might also be produced by specific bacterial classes. Eight-carbon-containing volatiles are important contributors to fungal aroma and have a characteristic mushroom flavor (43).

The remaining less common alcohol, ketone, ester, and aldehyde odorants found in truffle fruiting bodies have not been detected in truffle mycelia and are potentially produced only by guest filamentous fungi, yeasts, and/or bacteria. This is the case, for example, with 3-hydroxybutan-2-one, which potentially is produced by fungi of the *Sordariomycetes* class or the *Betaproteobacteria* class, which is a dominant group in the truffle microbiome (Fig. 1). Other rare volatiles might not be produced by microbes or by axenic cultures of truffle mycelia. It can be hypothesized that they are specific to the sexual stage of truffle fruiting bodies.

Similar to sulfur volatiles, the trend with alcohols, esters, ketones, and aldehydes is that common volatiles might be of mixed origins, while more specific ones might be produced either by microbes or truffles.

AROMATIC COMPOUNDS

Aromatic odorants produced by truffles include, for example, the volatile 2-phenylethanol with a characteristic rose smell, and benzaldehyde, an odorant with a characteristic bitter almond flavor (Fig. 2). Aromatic odorants might be derived from the catabolism of phenylalanine (36). Interestingly, with the exception of benzaldehyde and 2-phenylethanol, none of these volatiles have been detected in truffle mycelia (Fig. 2). These common aromatic odorants are potentially also synthesized by numerous fungal and or bacterial phyla and might therefore be of mixed origin (Fig. 2). The less common aromatic odorant 2-methoxy-4-methylphenol is potentially produced by the two bacterial classes *Bacilli* and *Gammaproteobacteria*, whereas some rare odorants might be derived from the sexual stage of truffles.

Overall, common aromatic odorants might be of either mixed fungal (truffle) or only microbial origin. The absence or rare occurrence in microbes of specific aromatic odorants suggests that they might be synthesized by truffles only and possibly during their sexual stage only.

OTHER VOLATILES

Butanoic acid and 4-hydroxy-2,5-dimethylfuran-3-one are odorants specific to *T. melanosporum* and *T. aestivum* (29). Based on what is seen in Fig. 2, they have been detected neither in truffle mycelia nor, in the case of 4-hydroxy-2,5-dimethylfuran-3-one, in microbes, which suggests that the latter volatile might be synthesized only during the sexual stage of truffles. Numerous microbes have the ability to produce butanoic acid, suggesting that it might be of microbial origin in truffle fruiting bodies (Fig. 2).

Overall, we are well aware that the absence of evidence is not evidence of absence. In other words, not having detected a volatile in a given organism does not demonstrate that the organism in question is not able to produce it under specific circumstances.

For example, this might be the case with truffles, which might produce specific odorants during their sexual stage only (fruiting body) and not as free-living mycelia (axenic cultures). Our approach, nevertheless, allows the construction of a hypothesis on the identity of the possible producers of specific odorants. Demonstrating what produces what will not only require fully characterizing pathways leading to specific odorants in truffles and microbes but also microbe-free truffles to be obtained. This is an especially challenging task considering that to date, all truffle fruiting bodies harvested from the wild contain microbes, and microbe-free fruiting bodies cannot be obtained under axenic conditions.

DO TRUFFLES OR ACTUAL MICROBES ATTRACT ANIMALS?

Truffles are hypogeous fungi, meaning that they form their fruiting bodies below the soil surface. Since their belowground habitat prevents them from dispersing spores through the air/wind, truffles have developed intense aromas to attract small rodents and larger mammals. These animals eat fruiting bodies and subsequently disperse truffle spores through their feces. Mammals are not the only animals that are able to locate fruiting bodies belowground; a beetle (*Leiodes cinnamomea* Panzer) and a fly (*Suillia pallida*) can achieve the same. However, it remains unclear whether these insects participate in spore dispersal or whether they just feed on truffles (44, 45).

Mammals are able to locate truffles belowground due to the dimethyl sulfide emitted by fruiting bodies (46). Dimethyl sulfide is obviously not the only volatile that animals can smell, since, for example, dogs, like humans, are able to distinguish between truffle species. Nevertheless, besides dimethyl sulfide, species-specific attractants have not been identified in truffles, and the structures of the compounds that attract flies and beetles to truffles are not known (44). The question of what actually produces these attractants raises interesting hypotheses about multitrophic interactions. Indeed, dimethyl sulfide might be of mixed fungal (truffle) and bacterial origin, since truffle mycelia and *Alphaproteobacteria*, which are dominant in fruiting bodies, are able to produce it. Assuming that dimethyl sulfide is partially derived from bacteria would imply that bacteria participate in attracting mammals and small rodents to truffles. A similar case has actually been demonstrated for the fruit fly, *Drosophila melanogaster*, which is not attracted by fruit volatiles but rather by microbial volatiles emitted by the yeasts that colonize the surface of the fruit (47). Finding the answer to what produces truffle attractants will require microbe-free truffles to be obtained, and this has not yet been achieved.

CONCLUSION

Understanding to what extent the microbiomes of truffles participate in truffle aroma formation promises to be a complex and challenging task. Literature data on the ability of organisms to produce volatiles suggests that truffles and microbes might be able to produce common truffle odorants, whereas more specific compounds might be of microbial origin only. Disentangling what produces what within truffle fruiting bodies will require elucidation of the biosynthetic pathways for specific odorants and the use of innovative techniques to follow the fate of aroma precursors *in situ*. Overall, truffles offer a unique opportunity to better understand the ecological function of microbes associated with fungi and their involvement in aroma formation.

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7.3 Chapter 3: Orchard conditions and fruiting body characteristics drive the microbiome of the black truffle *Tuber aestivum*

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RS: 100%

(2) Conducting tests and experiments

MV: 30%; RS: 30%; all other co-authors: 40%

(3) Compilation of data sets and figures

MV: 30%; all other co-authors: 70%

(4) Analysis and interpretation of data

MV: 20%; all other co-authors: 80%

(5) Drafting of manuscript

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Orchard Conditions and Fruiting Body Characteristics Drive the Microbiome of the Black Truffle *Tuber aestivum*

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Truffle fungi are well known for their enticing aromas partially emitted by microbes colonizing truffle fruiting bodies. The identity and diversity of these microbes remain poorly investigated, because few studies have determined truffle-associated bacterial communities while considering only a small number of fruiting bodies. Hence, the factors driving the assembly of truffle microbiomes are yet to be elucidated. Here we investigated the bacterial community structure of more than 50 fruiting bodies of the black truffle *Tuber aestivum* in one French and one Swiss orchard using 16S rRNA gene amplicon high-throughput sequencing. Bacterial communities from truffles collected in both orchards shared their main dominant taxa: while 60% of fruiting bodies were dominated by α-Proteobacteria, in some cases the β-Proteobacteria or the Sphingobacteriia classes were the most abundant, suggesting that specific factors (i.e., truffle maturation and soil properties) shape differently truffle-associated microbiomes. We further attempted to assess the influence in truffle microbiome variation of factors related to collection season, truffle mating type, degree of maturation, and location within the truffle orchards. These factors had differential effects between the two truffle orchards, with season being the strongest predictor of community variation in the French orchard, and spatial location in the Swiss one. Surprisingly, genotype and fruiting body maturation did not have a significant effect on microbial community composition. In summary, our results show, regardless of the geographical location considered, the existence of heterogeneous bacterial communities within *T. aestivum* fruiting bodies that are dominated by three bacterial classes. They also indicate that factors shaping microbial communities within truffle fruiting bodies differ across local conditions.

Keywords: *Tuber aestivum*, amplicon sequencing, bacterial communities, microbiome, multilocus genotype, mating type

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INTRODUCTION

Truffles are ascomycete ectomycorrhizal fungi that associate with the roots of a large number of trees and shrubs and that produce hypogeous fruiting bodies. Some truffle species such as *Tuber melanosporum* (Périgord black truffle) and *T. aestivum* (Burgundy truffle) are renowned worldwide for their delicate aroma and are considered as culinary delicacies. Although these truffles can be harvested in wild forests, over 80% of the truffles harvested in France are nowadays originating from artificially inoculated orchards (Murat, 2015). In this context, the controlled production of truffles is an economically important goal of research. Major progress has been made over the past 30 years to improve methods of truffle cultivation and to better understand the life cycle of these peculiar fungi (Paolocci et al., 2006; Rubini et al., 2007, 2011a,b; Murat, 2015; Molinier et al., 2016). The most comprehensive knowledge about truffle biology exist about *T. melanosporum* (Rubini et al., 2014; Le Tacon et al., 2016; Selosse et al., 2017) which was the first *Tuber* genome to be sequenced (Martin et al., 2010). However, mounting evidence based on genetics of *T. aestivum* (Molinier et al., 2013a,b, 2016) and the genomics of *T. aestivum* and *T. magnatum* suggests high similarities in terms of life cycle to *T. melanosporum* (Murat et al., 2018). In truffles, the life cycle starts with the germination of haploid spores. Hyphae produced from germinated spores colonize the fine roots of host plants and form ectomycorrhizae. This symbiotic mixed organ is the place of nutrients exchange between the two mutualistic partners (Smith and Read, 2008). Ectomycorrhizae also provide the maternal mycelium that will give birth to the fruiting body (or ascocarp) after mating with a paternal gamete of opposite mating type (Rubini et al., 2014; Selosse et al., 2017; Murat et al., 2018). In contrast to many other ectomycorrhizal fungi that produce fruiting bodies within a few days, the development of truffle fruiting bodies generally takes several months and occurs entirely belowground. In the case of *T. melanosporum*, it has been demonstrated that nutrients required for the development of the fruiting bodies are provided by the host plant all along fruiting body genesis (Le Tacon et al., 2013, 2015) and a similar process likely occurs for *T. aestivum* (Deveau et al., 2019). The production of fruiting bodies in all *Tuber* species varies greatly from year to year, ranging from none to several per tree. Additionally, considering trees with a sufficient degree of mycorrhization with *T. aestivum* or *T. melanosporum*, the yield of harvested truffles was shown to be unrelated to the host tree mycorrhization degree (Molinier et al., 2013a; De la Varga et al., 2017).

Beside the symbiotic association between the fungus and its host, it is now clear that complex microbial communities interact with truffle fungi both in the ectomycorrhizosphere and in the ascocarp. Based on a number of studies on truffle-associated bacterial communities, we know that the surface (peridium) and the inner tissues (gleba) of truffle fruiting bodies are colonized by complex bacterial communities composed of a few hundreds of species that can reach up to 10^7–10^8 cells per gram of truffle (Barbieri et al., 2007; Antony-Babu et al., 2014; Vahdatzadeh et al., 2015). The effects of these bacteria and of their interactions on the biology of truffles are still poorly understood. Yet, some bacteria have been shown to participate in the elaboration of some of the volatile organic compounds produced by the whitish truffle *Tuber borchii* (Splivallo et al., 2015), and it has been hypothesized that bacteria could be involved in the elaboration of the complex aroma of truffles (Vahdatzadeh et al., 2015). In addition, some bacteria of the *Bradyrhizobiaceae* family isolated from *T. magnatum* have shown the ability to fix nitrogen (Barbieri et al., 2010). It has been proposed that they could participate in the nutrition of the fungus during fruiting body development (Barbieri et al., 2010). Additional putative effects such as inhibition of pathogenic fungi, stimulation of the growth of *Tuber* mycelium, and ascocarp degradation have also been suggested based on potential functional activities of bacteria isolated from fruiting bodies (Citterio et al., 2001; Sbrana et al., 2002; Dominguez et al., 2012; Gryndler et al., 2013, 2015; Antony-Babu et al., 2014; Saidi et al., 2015; Deveau et al., 2016).

Despite differences between truffle species (Benucci and Bonito, 2016), the truffle microbiome is commonly dominated by bacteria belonging to the *Rhizobiales* order together with, to a lesser extent, members of the orders Actinomycetales, Burkholderiales, Enterobacteriales, Flavobacteriales, and Pseudomonadales (Barbieri et al., 2016). Yet, important variations in the composition of truffle microbiomes have been reported (Barbieri et al., 2016). Part of the discrepancies may be explained by the evolution of methodologies used to study microbial diversity, which cover from culture-dependent to various generations of culture-free methodologies (Sbrana et al., 2002; Barbieri et al., 2010; Deveau et al., 2016). Another part of this variability could be due to natural variation in microbiome composition among fruiting bodies of single *Tuber* species. Among the different factors that could influence truffle microbiome composition, the level of fruiting body maturation has been proposed as a potential driver of the microbiome composition in *T. borchii*, *T. indicum*, and *T. melanosporum* (Citterio et al., 2001; Antony-Babu et al., 2014; Splivallo et al., 2015; Ye et al., 2018). However, the extent to which other intrinsic (i.e., maturity, genotype, mating type) and extrinsic (i.e., season, location, spatial distance) factors drive the truffle microbiome is not known.

In this study, we filled this gap in knowledge by analyzing and comparing the microbiomes of more than 50 fruiting bodies of *T. aestivum* harvested over several years in two spatially distant orchards in Europe. *T. aestivum* is harvested and cultivated in numerous regions of the world (i.e., all over Europe, in Iran, Northern Africa) and its microbiome has not been extensively studied despite the fact that it represents one of the most relevant truffles in terms of traded volumes. We hypothesized that the microbial communities of *T. aestivum* would be dominated by bacteria of the *Bradyrhizobiaceae* family as in other truffle species but also that noticeable differences in microbial assemblages would be detectable between the two study sites due to variable environmental factors. To test and answer those hypotheses, (1) the "core" composition of the *T. aestivum* microbiome in both study sites was defined, (2) the variability in the truffle microbiome across orchards was assessed, and (3) the intrinsic factors (maturity, genotype, mating type) and extrinsic ones (season, location, spatial

distance) determining the assembly of truffle-associated bacterial communities were evaluated.

MATERIALS AND METHODS

Biological Material and Sampling

Fruiting bodies of *Tuber aestivum* (Vittad.) were collected from two artificially inoculated truffle orchards in France (FR) and Switzerland (SW). Exact GPS coordinates are not given here at the request of the orchard's owners, but the closest city nearby is provided as an approximate location. These orchards have been described earlier (Splivallo et al., 2012; Molinier et al., 2013a, 2015). The French orchard, located near Daix/Dijon (FR), is a 30-year old truffle orchard that comprises two rows of inoculated hazels (*Corylus avellana*) at its center and two outer rows of fruit trees on the outer margins (see for details Molinier et al., 2013a). All hazel trees in the French orchard were inoculated with *T. melanosporum* in 1976 and produced *T. melanosporum* fruiting bodies for nine seasons from 1980 to 1989 (a few hundred grams to 12 kg per year; Molinier et al., 2013a). During 1990–1993 production was gradually and eventually fully replaced by native *T. aestivum* and in subsequent years, production ranged from a few hundred grams to a few kilograms of *T. aestivum* per year (Molinier et al., 2013a). The soil of the French truffle orchard has a calcareous nature and a pH of 7.9 (Molinier et al., 2013a). The Swiss orchard, located in Valais, near St-Triphon (CH), contains 42 trees – oaks (*Quercus robur*) and pines (*Pinus nigra*) that were commercially inoculated with *T. aestivum* and planted in 1999. In this orchard, pH of the soil is 7.6 and production of *T. aestivum* started in 2008/2009 and ranged since then to a few hundred grams to approximately 1 kg per year.

A total of 62 *T. aestivum* fruiting bodies were collected from the two artificially inoculated truffle orchards. Seventeen truffles were collected in the French orchard in 2010 and 2011, whereas 45 truffles were collected from the Swiss orchard during four consecutive years (2009–2012). The precise location of truffles was recorded at the time of the harvest (**Figure 1**). To avoid post-harvest drifts of microbial populations, all truffles were cooled to 4°C after collection and frozen to −20°C within 24 h for subsequent DNA extraction.

Truffles were identified by spore morphology and via molecular methods (see the section "DNA Extraction and Truffle Genotyping"). The stage of fruiting body maturation was determined by estimating the percentage of ascii containing melanized spores, as previously described (Splivallo et al., 2012). An overview of the samples used in this study along with the analyses performed is shown in **Table 1**.

DNA Extraction and Truffles Genotyping

Genomic DNA was extracted from the gleba (50–100 mg fresh weight excised from the central part of the gleba) of each fruiting body using the DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Even though this kit might have been used here for characterizing truffle's microbiome for the first time, earlier works have demonstrated that various DNA extraction methods yielded comparable microbiome compositions for different truffle species (Antony-Babu et al., 2013; Benucci and Bonito, 2016). DNA qualities and concentrations were checked using a NanoDrop spectrometer and gel electrophoresis. Mating type identification was performed using the specific primers aest-MAT1-1f/aest-MAT1-1r and aest-MAT1-2f/aest-MAT1-2r as described elsewhere via multiplex polymerase chain reaction (PCR) (Molinier et al., 2016). In short, PCRs were carried out using 3 μl of template DNA (diluted 10 times) in a final volume of 20 μl containing 10 μl of JumpStart REDTaq ReadyMix (Sigma-Aldrich: P1107), 0.4 μl of each primer (0.2 μM each), and water to adjust to the final volume. Thermal cycles were conducted using the following program: an initial denaturation of 2 min at 94°C, 28 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min, followed by 72°C for 7 min. PCR products were checked on a 1.5% agarose gel and visualized after ethidium bromide staining by a UV transilluminator.

A total number of 14 SSR loci (aest01, aest06, aest07, aest10, aest15, aest18, aest24, aest25, aest26, aest28, aest29, aest31, aest35, and aest36) (Molinier et al., 2013b) were chosen for genotyping. The genotyping procedure followed that described by Molinier et al. (2016) but with a slightly modified PCR program: one cycle of 15 min at 95°C, 30 cycles of 30 s at 94°C, 90 s at 60°C, and 60 s at 72°C, and a final elongation cycle of 30 min at 60°C. To identify multilocus genotypes (MLGs) and true clones based on the 14 SSR markers, the software MLG_{SIM} (Stenberg et al., 2003) was used as described elsewhere (Molinier et al., 2016).

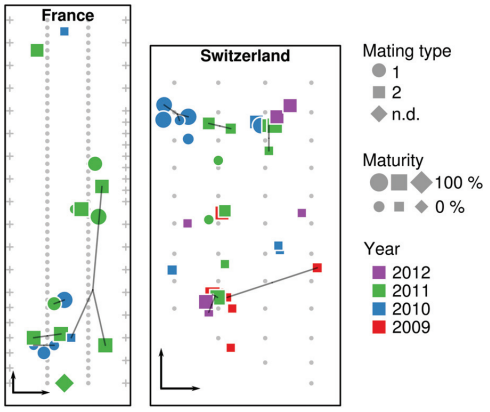

FIGURE 1 | Location of truffle samples within the French and Swiss orchards. Location of truffles, their maturity, mating type, collection year, and identical multilocus genotypes (MLGs, connected by lines) are shown in the French and Swiss orchards, along the position of truffle-mycorrhized trees (small gray dots) and fruit trees surrounding the orchard (crosses). Black arrows in the lower left corner of each orchard represent a distance of 5 m.

Microbiome Analysis

Bacterial communities of Swiss fruiting bodies were analyzed by 454 pyrosequencing, while French samples were analyzed by MiSeq Illumina sequencing, because 454 pyrosequencing

TABLE 1 | Details of the *Tuber aestivum* samples originating from the Swiss and French orchard, and results of bacterial microbiome analysis.

							Microbiome analysis					
Sample code	**Collection year**	**Collection month**	**Maturity (0% = fully immature, 100% = fully mature)**	**Genotype (MLG)* CH = Swuitzerland FR = France**	**Mating type**	**Sequencing technique (454 or MiSeq)**	**Number of sequencing reads before processing**	**Number of reads post processing**	**Number of OTU†**	**Shannon**	**Effective number of species**
13	2009	October	0%	CH_27	2	454	15,204	7,149	26	0.288	1
14	2009	October	0%	CH_29	2	454	17,689	7,058	8	0.394	1
15	2009	October	0%	CH_27	2	n/a	n/a	n/a	n/a	n/a	n/a
17	2009	October	0%	CH_22	2	454	27,548	12,868	26	0.672	2
18	2009	October	95%	CH_22	2	n/a	n/a	n/a	n/a	n/a	n/a
19	2009	October	11%	CH_22	2	454	17,645	8,280	65	2.075	8
20	2009	October	0%	CH_28	2	454	26,269	11,859	6	0.268	1
21	2009	October	0%	CH_27	2	n/a	n/a	n/a	n/a	n/a	n/a
24	2009	December	93%	CH_5	2	454	9,643	4,561	45	0.648	2
25	2009	December	54%	n/a	2	454	44,804	22,909	27	0.196	1
26	2009	December	73%	CH_17	2	454	22,250	10,740	53	1.350	4
27	2010	September	92%	CH_33	1	454	18,690	8,894	77	2.403	11
28	2010	September	2%	CH_31	1	n/a	n/a	n/a	n/a	n/a	n/a
29	2010	September	0%	CH_3	2	454	15,096	7,390	26	0.204	1
30	2010	September	87%	CH_34	1	454	27,672	12,367	17	1.061	3
31	2010	September	1%	CH_34	1	454	19,699	9,673	23	0.862	2
32	2010	September	84%	CH_8	1	454	14,311	6,821	4	0.679	2
34	2010	September	82%	CH_7	1	454	24,439	10,966	9	0.548	2
40	2010	September	15%	CH_20	2	454	10,840	4,965	34	0.886	2
43	2010	September	69%	CH_11	2	454	9,640	4,303	14	0.071	1
44	2010	December	96%	CH_34	1	454	32,011	14,956	6	0.750	2
45	2010	December	0%	CH_2	2	454	24,123	11,645	54	0.755	2
47	2010	December	13%	CH_31	1	n/a	n/a	n/a	n/a	n/a	n/a
48	2010	December	19%	CH_32	1	454	26,987	13,060	5	0.140	1
49	2010	December	0%	CH_25	1	n/a	n/a	n/a	n/a	n/a	n/a
50	2010	December	25%	CH_16	1	n/a	n/a	n/a	n/a	n/a	n/a
51	2010	December	85%	CH_19	1	n/a	n/a	n/a	n/a	n/a	n/a
52	2010	December	86%	CH_24	2	n/a	n/a	n/a	n/a	n/a	n/a
53	2011	August	55%	CH_18	2	454	28,556	13,665	9	0.023	1
55	2011	August	30%	CH_18	2	454	12,310	6,054	56	2.300	10

(Continued)

TABLE 1 | Continued

						Microbiome analysis					
Sample code	**Collection year**	**Collection month**	**Maturity (0% = fully immature, 100% = fully mature)**	**Genotype (MLG)* CH = Swuitzerland FR = France**	**Mating type**	**Sequencing technique (454 or MiSeq)**	**Number of sequencing reads before processing**	**Number of reads post processing**	**Number of OTU†**	**Shannon**	**Effective number of species**
57	2011	August	0%	CH_13	2	454	16,204	8,140	12	0.062	1
59	2011	August	0%	CH_30	2	454	22,247	11,161	9	0.029	1
63	2011	November	70%	CH_9	2	454	14,862	6,325	9	0.329	1
65	2011	November	5%	CH_9	2	454	31,539	15,919	6	0.055	1
67	2011	November	0%	CH_23	1	454	27,997	13,450	7	0.249	1
69	2011	November	95%	CH_21	2	454	18,199	9,109	7	0.024	1
71	2011	November	95%	CH_5	2	454	18,022	9,271	37	0.340	1
73	2011	November	50%	CH_15	2	454	20,369	9,762	12	0.042	1
75	2011	November	0%	CH_14	1	454	15,607	7,883	9	0.070	1
77	2012	August	75%	CH_12	2	454	19,118	9,791	22	0.158	1
78	2012	August	75%	CH_10	2	454	14,444	6,658	24	1.816	6
79	2012	August	5%	CH_27	2	454	24,087	12,414	28	0.236	1
80	2012	December	1%	CH_26	2	454	24,658	12,032	15	0.101	1
82	2012	December	85%	CH_6	2	454	21,038	9,811	13	0.052	1
84	2012	December	0%	CH_1	2	454	23,360	10,788	16	0.599	2
D1	2010	October	0%	FR_23	n/a	MiSeq	33,632	26,296	97	2.613	13
D2	2010	October	7%	FR_23	1	MiSeq	44,386	41,798	91	1.300	4
D3	2010	October	44%	FR_2	1	MiSeq	46,082	43,159	109	1.213	3
D4	2010	October	0%	FR_12	2	MiSeq	37,830	33,175	83	1.484	4
D5	2010	October	0%	FR_10	2	MiSeq	37,405	36,450	75	0.401	2
D21	2010	December	94%	FR_20	n/a	MiSeq	37,956	35,472	57	2.113	8
D25	2011	October	0%	FR_22	1	MiSeq	38,708	38,159	53	0.178	1
D26	2011	October	70%	FR_15	2	MiSeq	37,358	36,499	55	0.301	1
D27	2011	October	90%	FR_6	2	MiSeq	40,495	35,131	96	1.700	6
D28	2011	October	88%	FR_18	1	MiSeq	40,245	38,394	68	0.615	2
D29	2011	October	92%	FR_12	2	MiSeq	39,319	38,719	49	0.224	1
D30	2011	October	50%	FR_20	1	MiSeq	39,187	38,520	37	0.086	1
D31	2011	October	87%	FR_8	2	MiSeq	38,060	36,839	34	0.644	2
D32	2011	October	91%	FR_8	2	MiSeq	39,274	38,597	36	0.194	1
D34	2011	October	65%	FR_5	1	MiSeq	31,638	28,588	60	2.075	8
D35	2011	November	81%	FR_4	n/a	MiSeq	39,007	37,880	69	0.302	1
D36	2011	November	82%	FR_12	n/a	MiSeq	40,582	39,764	46	0.167	1

**MLGs are named as CH for Switzerland and FR for France followed by a number. Note that the same number (i.e., CH_5 and FR_5) do not refer to the same MLG as the MLG analysis for both orchards were done independently from each other. †Number of OTUs after singleton removal and elimination of rare OTUs (OTUs with a total number of reads inferior to 0.01% of the total number of all samples). n/a, missing data.*

technique was no longer available at the time of the analysis. In both cases, the isolated DNA from the gleba of fruiting bodies was used to generate 16S rRNA gene amplicon libraries using the primers 787r (ATTAGATACCYTGTAGTCC) (Nadkarni et al., 2002) and 1073F (ACGAGCTGACGACARCCATG) (On et al., 1998), modified to include specific linkers and identification barcode sequences for the respective sequencing method. The same procedure as described by Antony-Babu et al. (2014) was used to generate 454 pyrosequencing amplicons. Briefly, the PCRs contained 10 μl of PCR Mastermix (5 PRIME), 1 μl of each forward and reverse primers (each 0.2 μM), and 2 μl of template DNA (or sterile water for negative control) in a final volume of 25 μl. For each truffle DNA sample, amplifications were performed in three parallel PCR tubes under the following conditions: an initial denaturation at 94°C for 10 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 48°C for 45 s, extension at 72°C for 90 s, and a final extension at 72°C for 10 min. The three PCR products were pooled and quantified by gel electrophoresis and an equimolar mix of amplicons was used for pyrosequencing. Amplicon sequencing was performed by the GS-FLX 454 Titanium platform of Beckman Coulter Genomics (Danvers, MA, United States). Illumina MiSeq amplicons were produced using the same amplification protocol except that the identification barcode sequences were added through a second round of amplification as described by Barret et al. (2015). PCR cycling conditions were 94°C for 2 min, followed by 12 cycles of amplification at 94°C for 1 min, 55°C for 1 min, and 68°C for 1 min each, and a final extension step at 68°C for 10 min. All amplicons were purified with the Agincourt AMPure XP system and quantified with QuantIT PicoGreen. The purified amplicons were then pooled in equimolar concentrations, and the final concentration of the library was determined using a quantitative PCR (qPCR) next-generation sequencing (NGS) library quantification kit (Kapa Biosystems, Boston, MA, United States). Amplicon libraries were mixed with 10% PhiX control according to the 2 × 250 bp Illumina protocols. The second round of PCRs, the purification steps, and sequencing was performed by the GeT PLAGE sequencing platform according to standard procedures (INRA Toulouse). The standard procedure to generate libraries for Illumina Miseq is available here: https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf.

Both 454 pyrosequencing and MiSeq Illumina 16S rRNA sequences were analyzed using FROGS (Escudié et al., 2018). After quality control and demultiplexing, sequences were preprocessed by removing primers from sequences, sequences out of the amplicon size range (250–300 bp), sequences with only one primer, with at least one homopolymer longer than 7 bp and a Phred quality score <10, and replicates of identical sequences. For the MiSeq Illumina run, 16S rRNA paired-end sequences were first merged (289 bp). Sequences were clustered into operational taxonomic units (OTUs) at 97% sequence similarity based on the iterative Swarm algorithm, with subsequent removal of chimeras for further analysis. Taxonomy assignment to each cluster was carried out by BLAST comparisons against the SILVA database and using the RDP Classifier (Ribosomal Database Project; Cole et al., 2009). OTUs with poor affiliation or higher abundance in negative controls than samples were deleted for further analysis. Finally, OTUs with a total number of reads inferior to 0.01% of the total number of all samples were discarded. The raw data are deposited in the NCBI Sequence Read Archive website[1] under the BioProject study accession number PRJNA506316 and the SRA accession numbers SRX5059925–SRX5059959 (454 sequences) and SRX5073276–SRX5073292 (Illumina sequences).

Statistical Analyses

All statistical analyses were performed in R (R Core Team, 2017) with the aid of relevant packages. The datasets from FR and SW were processed independently, but using the same procedure. The datasets were only combined to generate a joint heat tree using the R package Metacoder v0.1.3 (Foster et al., 2017), to summarize the overall taxonomic composition obtained and compare the relative proportion of taxa between both studies. Differences between sites in the relative abundances of the main bacterial taxa were assessed via one-way ANOVA followed by Tukey's HSD *post hoc* test, after verifying normality of data with Shapiro–Wilk test. Overall and per-sample rarefaction curves were calculated in each dataset to assess sampling completeness, using function *rarecurve()* in package Vegan v3.5-1 (Oksanen et al., 2015). Based on these, subsequent analyses of diversity and community structure were performed on datasets where samples had been rarefied with the Phyloseq package (McMurdie and Holmes, 2013) to achieve equal read numbers of 26,295 for the FR dataset and 3,855 for the SW dataset. Rarefaction curves were used to verify that the subsampling was performed as close as possible to the asymptotes to allow comparison between samples in both French and Swiss sites (**Supplementary Figure 1**). Values of OTU richness and diversity based on Shannon's index were calculated using functions available in Vegan. Effective numbers of species were calculated using Simpson index as proposed by Jost (2006).

Non-metric multidimensional scaling (NMDS) was used to visualize differences in community composition among samples. NMDS is an ordination method that represents pairwise (dis)similarities between samples in a low-dimensional space, so that samples placed closer in the graph are more similar than those further apart (Clarke, 1993; Legendre and Legendre, 2012). NMDSs were based on Bray–Curtis dissimilarities calculated among samples after a Hellinger transformation of data (Legendre and Gallagher, 2001).

We investigated the potential influence of factors on microbiome variation using variation partitioning based on distance-based redundancy analysis (db-RDA; Borcard et al., 2011; Legendre and Legendre, 2012), with the Hellinger-transformed dissimilarity matrix as response variable. db-RDA is a constrained ordination method in which a matrix of pairwise (dis)similarities between samples is modeled as a function of a set of explanatory variables (Legendre and Legendre, 2012). Variation partitioning

[1] http://www.ncbi.nlm.nih.gov/sra

can then be applied to measure the relative contribution of individual explanatory variables to overall community variation while accounting for the effects of other variables, by sequentially removing components from the db-RDA model and recording the resulting changes in the total variance explained.

The explanatory variables to be included in the variation partitioning analysis were selected, when possible, by means of permutational analysis of variance (PERMANOVA; Anderson, 2001), so that only those with a significant correlation with community variation ($P < 0.05$) in at least one dataset were retained. The potential influence on community structure of spatial distance among samples was first examined using Mantel correlograms (Legendre and Legendre, 2012), which enable to test whether samples that are spatially close are more similar than those farther apart. Then, to allow their inclusion in the variation partitioning analysis, the spatial relationships were summarized as principal coordinates of neighbor matrices (PCNM) vectors (Legendre and Legendre, 2012), calculated from the coordinates of each sample within the orchard using the function *pcnm()* of vegan. PCNMs describe non-random patterns in dissimilarity matrices at different scales, which can then be used to model potential sources or variation not accounted for by the measured explanatory variables, such as dispersal, species interactions, or historical causes (Peres-Neto and Legendre, 2010). PCNM vectors significantly associated with community variation in our datasets were forward-selected using package Packfor v0.0-8 (Dray et al., 2009). Because no PCNMs were significantly correlated with the FR dataset, in this case, we manually selected the first four to match the number of PCNMs retained for SW. As done with spatial distances among samples, the factor truffle genotype was assessed by summarizing Euclidean distances among SSR profiles with PCNM vectors and testing their association with microbiome variation by forward selection. After the selection of factors, the final db-RDA models included as explanatory variables truffle mating type, degree of maturity, year of collection, and spatial distance. Truffle maturity and SSR genotypes were excluded because they did not explain an important nor significant amount of microbiome variation in any location.

RESULTS

High-Throughput Sequencing

A total of 661,164 and 757,177 quality-passed sequences were obtained for the French (Illumina sequencing) and Swiss (454 sequencing) orchards, respectively, with averages of 38,892 (±807 SE) and 21,033 (±1,209) reads per sample (**Table 1**). After quality filtering and removal of chimeric reads, a total of 623,440 and 362,697 sequences were retained, with an average of 36,673 (±1,023) and 10,075 (±610) reads per sample across the French and Swiss samples, respectively (**Table 1**). After taxonomy assignment, elimination of contaminants and of OTUs present in <0.01% of the total number of reads (128 and 1,177 OTUs, respectively), 183 and 147 OTUs were considered for further analyses in the French and Swiss samples, respectively.

Truffles From Two Distant Orchards Have Similar Microbiomes

We first compared the microbiome composition of truffles collected from the French and Swiss orchards. An average of 66 ± 6 (SE) OTUs were detected in the French samples, while 23 ± 3 were recorded in average in the Swiss ones. This important difference could be a bias due to the use of MiSeq Illumina sequencing (French orchard) and 454 pyrosequencing (Swiss orchard). Indeed, Illumina sequencing allows for larger numbers of reads per sample and may provide a better access to rare OTUs. This hypothesis was confirmed on another set of data obtained from *T. melanosporum* fruiting bodies analyzed both by 454 and MiSeq Illumina sequencing (Deveau et al., unpublished data). The two samples that were analyzed by both methods strongly differed in richness (21 vs. 98 OTUs for 454 and Illumina MiSeq, respectively) but the relative abundance of the dominant genera that were found in this study was similar no matter which methodology was used (**Supplementary Figure 2**). In accordance with this observation and despite the two different sequencing methods used, the general composition of the truffle microbiomes detected at each site was alike, as shown in **Figure 2**. In both locations, communities were dominated by OTUs affiliated to the α-Proteobacteria (FR: 67 ± 9% SE, CH: 63 ± 7%; $P > 0.05$) followed by closely related proportions of Bacteroidetes (FR: 9 ± 4%; CH: 14 ± 5%; $P > 0.05$), β-Proteobacteria (FR: 9 ± 6%, CH: 17 ± 5%; $P > 0.05$), and γ-Proteobacteria (FR: 10 ± 4%, CH: 4 ± 2%; $P > 0.05$). Overall, Actinobacteria (FR: 2.5 ± 0.7%, CH: 0.6 ± 0.2%; $P < 0.01$) and Firmicutes (FR: 2.7 ± 1.7%, CH: 0.1 ± 0.02%; $P > 0.05$) were less frequent, with Actinobacteria being the only phylum with a significantly different abundance between the two orchards. OTUs of d-Proteobacteria, Acidobacteria, and Verrucomicrobia were found at very low levels in some samples of the two sites. Strong similarities between the two orchard's samples were also observed at the genus level, since the most represented OTUs belonged to the same genera: *Bradyrhizobium* (FR: 65.1 ± 8.8%, CH: 58.6 ± 6.9%), *Pseudomonas* (FR: 8.1 ± 3.4%, CH: 3.4 ± 1.4%), *Pedobacter* (FR: 4.3 ± 3.3%, CH: 13.8 ± 4.9%), *Polaromonas* (FR: 5.4 ± 5.0%, CH: 9.2 ± 4.4%), and *Flavobacterium* (FR: 2.5 ± 1.2%, CH: 0.8 ± 0.7%). Twenty-three additional genera were shared between the two datasets. This "core" microbiome contained genera belonging to five different Phyla (**Supplementary Table 1**). Differences nevertheless also existed between the two localities at the genera level as several dozens of genera were also specifically found on one of the two orchards. Yet it is here difficult to discriminate the part of sequencing methodology bias from true data.

α-, β-Proteobacteria, and Sphingobacteriia Dominate Single Fruiting Bodies

Having compared the overall microbiomes of the French and Swiss orchards, our next aim was to assess the variability in

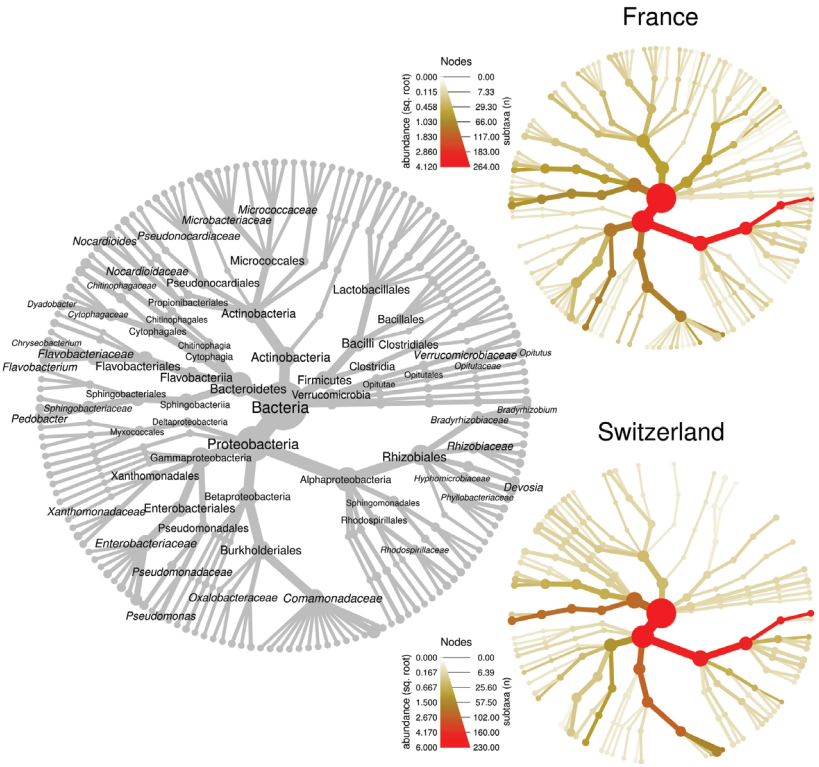

FIGURE 2 | Bacterial community structure within *T. aestivum* fruiting bodies collected from two orchards. Each heat tree represents bacterial community structure as a taxonomic hierarchy up to genus level. The gray tree serves as a key for the smaller unlabeled trees, node labels highlight the most abundant taxa detected at both orchards. The smaller colored trees show community structure for each orchard, assessed with different amplicon sequencing technologies [MiSeq for France (FR), 454 for Switzerland (SW)]. Node and edge sizes are proportional to the number of OTUs within each taxon, whereas color represents taxon abundances (square root of read numbers).

bacterial community structure and taxonomic composition among fruiting bodies. The bacterial community structure and composition was highly variable among single fruiting bodies collected within the same orchard. In both the French and Swiss orchards, the number of OTUs detected per truffle samples varied from a few OTUs to more than a hundred (**Table 1**). Such variation was independent from the sequencing depth obtained for each sample (**Table 1**). It is thus likely not due to a bias of sequencing depth but rather reflects different patterns of bacterial community structures, some truffles being colonized by a small number of species while others harbored a larger number of species. The evenness of the bacterial communities also deeply differed between samples in both orchards as illustrated by the strong variability of the Shannon and the effective species value (**Table 1**). While most truffle-associated bacterial communities were dominated by a few abundant OTUs and numerous rare OTUs, a few fruiting bodies of both sites showed more balanced patterns (data not shown). Such heterogeneity was also reflected when looking at the composition of the bacterial communities at different taxonomic levels (**Figure 3**). Overall, at the phylum level, 57% of the fruiting bodies showed communities dominated by α-Proteobacteria while 13% of the fruiting body communities were dominated by β-Proteobacteria, and 11% by Bacteroidetes. Eight percent of the fruiting bodies harbored balanced communities in which several phyla were co-dominants. A similar pattern was maintained at the genus level, with *Bradyrhizobium* (α-Proteobacteria), *Polaromonas* (β-Proteobacteria), and *Pedobacter* (Sphingobacteriia) being the most abundant genera depending on the fruiting body considered. To a lesser extent, OTUs from the *Variovorax* genus (β-Proteobacteria), *Pseudomonas* (g-Proteobacteria), *Sphingomonas* (α-Proteobacteria), and *Flavobacterium* (Flavobacteria) formed a significant part of the communities in some fruiting bodies. Thus, the large sampling effort realized over several years in the two truffle orchards revealed the existence of an unsuspected important variability in the composition of the microbiome of truffle ascocarps.

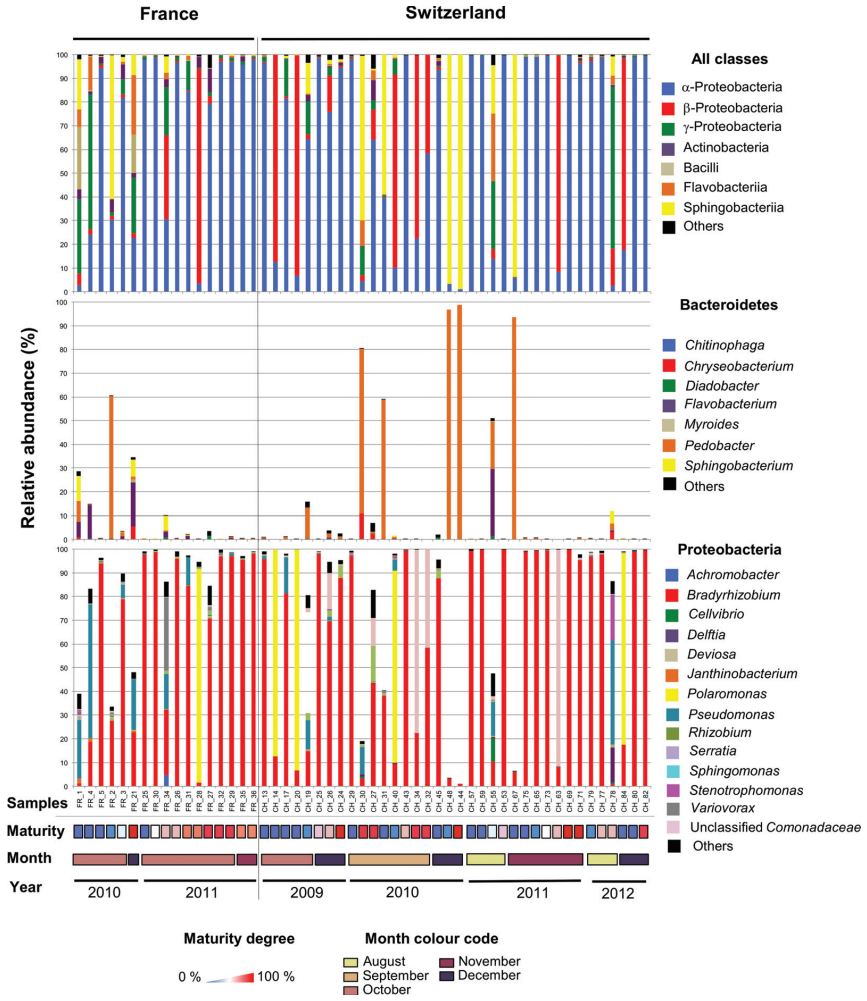

FIGURE 3 | The microbiome of *T. aestivum*. Distribution of major bacterial classes (top panel) and genera (intermediate and bottom panels) in the French and Swiss truffle fruiting bodies analyzed here. Each column represents a single sample. For each sample, three pile-up plots are given: the relative distribution of reads among the major bacterial classes (top panel) and of the different genera forms the Bacteroidetes (intermediate panel) and Proteobacteria phyla (bottom panel). Samples were ordered according to the year and month of collection, and maturity degree (from low to high).

Mating Type and Multilocus Genotype Distribution of Truffle Fruiting Bodies Within the Orchards

Truffle fruiting bodies result from the fertilization of two individuals of opposite mating type (Martin et al., 2010; Rubini et al., 2011b). Whereas the truffle gleba (maternal tissue) is made up by one individual, the spores contain meiotic products of the two mating partners (Paolocci et al., 2006; Rubini et al., 2011b; Selosse et al., 2017). Here, we determined the genetic profile of the truffle gleba (maternal genotype) only, since the gleba harbors most of the truffle microbiome (Antony-Babu et al., 2014; Splivallo et al., 2015).

Genotyping of the truffles from the French orchard had been done in a previous study (Molinier et al., 2016). A large proportion of unique genotypes (i.e., genotypes that were recorded only once) was observed: eight truffles had unique genotypes and only two pairs with the same MLG (here FR12 and FR20) were detected over the 2010–2011 seasons (**Table 1**). Truffles of opposite mating types appeared to be evenly spread over the French orchard. In the Swiss

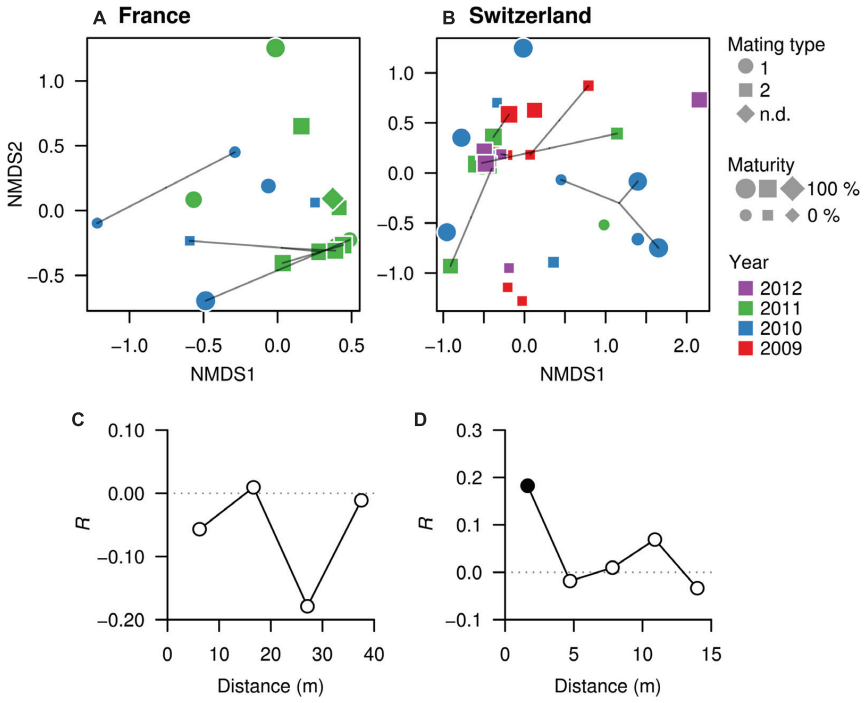

FIGURE 4 | Microbial community similarities among truffles within orchards. (**A**, FR; **B**, SW) Non-metric multidimensional scaling (NMDS) ordinations based on Bray–Curtis dissimilarities of the OTU composition of microbiomes in truffle samples. The closer the samples, the more similar their microbiomes are. Different symbols denote mating type, symbol sizes represent maturity of fruiting bodies, and symbol colors represent collection year. Points linked with lines are fruiting bodies belonging to the same MLG. (**C**, FR; **D**, SW) Correlograms showing correlation of microbiome similarity among samples (*y*-axis, Mantel's *R*) with spatial distance (*x*-axis). Solid and empty points denote significant ($P < 0.05$) and non-significant correlations for each distance class based on Bonferroni adjustment for multiple testing, indicating that space affects the truffles microbiomes in the Swiss but not the French site.

orchard, out of the 44 fruiting bodies for which the MLG was identified, 26 had unique MLGs, whereas other MLGs were shared among the remaining 18 samples. In particular, four MLGs (namely CH_5, CH_9, CH_18, and CH_31) were shared between two individuals, two (CH_22 and CH_34) among three individuals, and only one (CH_27) among four individuals (**Figure 1**). In terms of collection season, five out of seven shared MLGs were found in truffles harvested in the same season, while only two MLGs were shared by truffles harvested 2–3 years apart. Last and by contrast to the French orchard, truffles of mating type 1 were strongly aggregated in one corner of the Swiss field, whereas the rest of the orchard was dominated by truffles of mating type 2 (**Figure 1**).

Different Factors Affect Truffle's Microbial Communities in the French and Swiss Orchards

Having observed important differences in microbial community composition and structure within truffles of the same orchard, we explored whether this variability could be linked to a series of biotic and abiotic factors inherent to truffle ascocarps and to truffle orchards. Specifically, we considered seasonality, space (the location of truffles within an orchard), fruiting body genotype, mating type, and maturation as potential factors affecting the microbiome.

Non-metric multidimensional scaling was used to visualize pairwise dissimilarities between each truffle-associated microbiome and to explore their relationships with intrinsic (maturation, genotype, mating type) and extrinsic factors (collection season, year, or spatial distance) potentially explaining microbial community structure. NMDS ordinations based on Bray–Curtis dissimilarities among samples in each field showed no evident sample groupings related to truffle maturity, mating type, or MLGs (**Figures 4A,B**). However, in the Swiss orchard, mating type was significantly associated with microbiome variation based on PERMANOVA analysis (**Figure 4B**; $F_{(1,35)} = 4.6$, Adj. $R^2 = 0.12$, $P < 0.002$), whereas in the French field, a significant effect was found for the collecting year ($F_{(1,16)} = 3.2$, Adj. $R^2 = 0.17$, $P = 0.016$). Likewise, a different effect of spatial distance on bacterial communities was

TABLE 2 | Variation in microbial community composition explained by mating, year of collection, and space.

	France			Switzerland		
	df	**Percentage of variation explained (Adj. *R*-square)**	***P***	***df***	**Percentage of variation explained (Adj. *R*-square)**	***P***
Mating	1	0.03	0.154	1	**0.09**	**0.001**
Year	1	**0.11**	**0.010**	1	0.01	0.169
Space	4	−0.09	0.872	4	**0.18**	**0.001**
Residuals	n.d.	0.82	n.d.	n.d.	0.81	n.d.

For each orchard (FR, SW), the table lists the proportion of overall variation exclusively explained by each factor based on variation partitioning analysis of db-RDA models. Significant values ($P < 0.05$) are shown in bold typeface. The factors maturation and genotype were not included in this analysis since they were previously shown not to influence the microbiome composition. n.d. = not determined.

found in each site: whereas no association was found in the French orchard (**Figure 4C**), in SW, similarities among truffle microbial communities appeared to be significantly influenced by distance, with a strong aggregation pattern up to a distance of approximately 2 m (**Figure 4D**).

Distance-based redundancy analysis ordinations were applied to model the variation in truffle microbiomes in the French and Swiss orchards as a function of the explanatory variables that significantly influenced the microbiome: truffle mating type, year of collection, and spatial distribution of samples (PCNMs). Other factors (maturation and genotype) were excluded as they did not have a significant effect as demonstrated earlier (see also the section "Materials and Methods"). The db-RDAs models constrained by truffle mating type, year of collection, and spatial distribution explained 19% (F_2 = 2.3, P = 0.002) and 18% (F_2 = 1.5, P = 0.048) of overall community variation for the Swiss and French orchards, respectively. In the Swiss orchard, the associations of community structure with mating type and spatial factors previously reported were confirmed (**Figure 4B**), with spatial distance and mating type explaining an overall 18 and 9% of total variation, respectively, after accounting for the effects of other variables (**Table 2**). These values contrasted with a comparably low contribution (1.0%) of collection year (**Table 2**). In FR, the only variable with a significant correlation with microbiome structure was the collection year, with an overall 11% (P = 0.010) of the variance explained (**Table 2**).

DISCUSSION

Host-associated microbiomes are important for the nutrition and health of their hosts: plants, animals, and macrofungi are extensively colonized by microorganisms that play key roles in their life cycles (Berg et al., 2014; Bahrndorff et al., 2016; Webster and Thomas, 2016; Pent et al., 2018). Studies on animals and plants have revealed that host identity, genotype, and environmental variables all contribute to shaping the microbial communities colonizing eukaryotic tissues (Bulgarelli et al., 2012; Lundberg et al., 2012; Bouffaud et al., 2014; Hacquard et al., 2015; Glynou et al., 2016), but the relative importance of each factor varies depending on the host and on the type of environment. Fungi also host complex microbial communities that can associate to various fungal structures (i.e., mycorrhizas, mycelium, fruiting bodies) and colonize either the surface of hyphae or the intracellular compartments (Deveau et al., 2018). However, the factors that drive the assembly of these communities are poorly documented. A recent study on the microbiome structure of the epigeous fruiting bodies of the saprophytic fungus *Marasmius oreades* revealed that host genetic differences could be responsible for 25% of bacterial community structure variation (Pent et al., 2018). The authors thus proposed that, similarly to what's known for animals and plants, host genetics could be an important driver of the structure and function of the microbiome of fungal fruiting bodies (Bulgarelli et al., 2012; Chaston et al., 2016). This was however not the case in this study for *T. aestivum* suggesting that microbiome drivers might thus differ in different fungal species.

Unexpected Truffle Microbiome Variations Revealed Through Extensive Sampling

The relevance of truffle microbiomes lies in their involvement in aroma production (Splivallo et al., 2015; Splivallo and Ebeler, 2015; Vahdatzadeh et al., 2015) and impact on truffle's shelf-life/freshness (Rivera et al., 2010). We provide here the first extensive description of the microbiome of the summer black truffle *T. aestivum*. The overall structure of the bacterial communities observed in Swiss and French *T. aestivum* truffles corroborates earlier results obtained from other species of black and white truffles originating from Europe, China, and the United States (Antony-Babu et al., 2014; Barbieri et al., 2016; Benucci and Bonito, 2016; Ye et al., 2018). We confirmed that the *T. aestivum* fruiting body microbiome is characterized by an overall dominance of the α-Proteobacteria mainly affiliated to the *Bradyrhizobium* genus. However, unusual patterns were obtained for about 30% of the fruiting bodies from both Swiss and French truffle orchards. In these cases, microbiomes were dominated by OTUs affiliated to the genera *Pedobacter* (Bacteroidetes), *Polaromonas* (β-Proteobacteria), or *Pseudomonas* (γ-Proteobacteria), and not by α-Proteobacteria. The richness of the communities tended to be reduced to 10–20 OTUs when these genera dominated, suggesting that these particular genera massively invaded the gleba of the fruiting bodies and replaced or competed with *Bradyrhizobium*. By

contrast, a few fruiting bodies were characterized by quite diverse and even bacterial communities containing up to 100 different OTUs (e.g., FR_34, CH_55, CH_78). These different microbiome patterns and the occasional preponderance of particular taxa have so far not been reported for any white and black truffle species (Antony-Babu et al., 2014; Vahdatzadeh et al., 2015; Benucci and Bonito, 2016; Ye et al., 2018) but it is in agreement with the discrepancies noticed between studies performed on identical species by different research groups (Barbieri et al., 2016). These differences might be explained by the low numbers of fruiting bodies of diverse truffle species analyzed so far (a few fruiting bodies vs. >50 in our study). Such variability in community composition between fruiting bodies is likely not a specificity of *T. aestivum* truffles, as preliminary results obtained on a large survey of *T. melanosporum* suggest the same trend (Deveau et al., unpublished data).

The ecological function of bacteria colonizing truffle fruiting bodies remains speculative but it has been hypothesized that they might contribute to truffle nutrition as well as aroma variability (Barbieri et al., 2010; Splivallo et al., 2015; Splivallo and Ebeler, 2015; Vahdatzadeh et al., 2015). It is tempting to speculate that differences in microbial communities might explain variability in aroma documented for *T. aestivum* truffles collected from the same orchard (Splivallo et al., 2012; Molinier et al., 2015). However, aroma variability in *T. aestivum* was linked earlier to truffle genotype, yet genotype did not contribute in the present study to microbial community structuring. This suggests that microorganisms might after all not play a major in the aroma variability of *T. aestivum*. Clearly, this hypothesis will need to be tested in the future, for example, by characterizing the volatile profiles of single major OTUs in the presence of truffle substrate (Splivallo et al., 2015).

The data presented here highlight the importance of three bacterial genera in truffles, namely *Bradyrhizobium*, *Pedobacter*, and *Polaromonas*. Even though the specific functions of these genera in truffles remain to be demonstrated, it has been suggested that *Bradyrhizobium* could be involved in the nutrition of the fruiting bodies since the role of this genus as nitrogen-fixing symbionts is well established in plant roots (Sulieman and Tran, 2014; Coba de la Peña et al., 2018). Nitrogen fixation by *Bradyrhizobium* strains isolated from the white truffle *T. magnatum* has been previously detected (Barbieri et al., 2010), even though several lines of evidence suggest that this might not occur in the black truffle *T. melanosporum* (Barbieri et al., 2016; Le Tacon et al., 2016) where *Bradyrhizobium* strains might be missing the nifH genes involved in nitrogen fixation (Antony-Babu et al., 2014; Deveau et al., unpublished data). This corroborates the recent proposition based on genome comparisons that symbiosis was not the dominant lifestyle of *Bradyrhizobium* but rather on form of specialization (VanInsberghe et al., 2015). *Bradyrhizobium* might also be involved in the production of specific sulfur volatile compounds responsible of truffle aroma perceived by humans (Splivallo et al., 2015). Bacteria of the *Pedobacter* genus have been reported to dominate microbial communities of decomposing fungal mycelium in forest soil and litter (Brabcová et al., 2016). These bacteria regroup generalists that possess a wide array of enzymes allowing degradation of diverse carbon sources. Additionally, some *Pedobacter* produce chitinases to degrade chitin of fungal cell wells. Although no obvious sign of degradation of the fruiting bodies was visible in our samples at the time of harvest, it is tempting to speculate that these bacteria could participate to the degradation of truffle fruiting bodies. Last, the role played by *Polaromonas* in truffles remains elusive. The genus comprises nine commonly occurring species that were originally reported from cold environments. Some members of the *Polaromonas* have the ability to fix nitrogen, hydrogen, and carbon dioxide (Sizova and Panikov, 2007; Hanson et al., 2012), suggesting that they could have similar functions in truffles. Demonstrating the exact function in truffles of these three bacterial genera will be the focus of future work.

Site-Specific Factors Drive Truffle's Microbiome Assemblages

Multiple biotic and abiotic factors could drive the composition of the bacterial communities colonizing fruiting bodies of truffles. As the biochemical composition of fruiting bodies strongly changes during several months of maturation of *T. melanosporum* fruiting bodies (Harki et al., 2006), the level of maturity could be an important intrinsic driver of the bacterial communities. Indeed, a correlation was noticed between the bacterial community composition and the level of maturity of fruiting bodies of *T. borchii, T. melanosporum*, and *T. indicum* (Citterio et al., 2001; Antony-Babu et al., 2014; Ye et al., 2018). In contrast, the community composition of the white truffle *T. magnatum* remained stable along the maturation according to Barbieri et al. (2007). Such correlation between maturity degree and the composition of the microbiome was not evidenced in the present study, nor did we observe any link with the abundance of β-Proteobacteria or Bacteroidetes as previously reported by Antony-Babu et al. (2014) in *T. melanosporum*. Whether this is a specificity of *T. aestivum* remains to be determined. A possible reason might be the fact that *T. aestivum*, unlike *T. melanosporum* and other fungi, seems to pass through several lifecycles within a year with no clear seasonality, showing ripe and unripe fruiting bodies uncorrelated with size almost throughout a year (Büntgen et al., 2017). Such asynchrony of maturation might allow to more clearly disentangle maturation from spatial and temporal effects on bacterial communities in truffles. In agreement with this hypothesis, our data highlight a significant contribution of the spatial distance (Swiss orchard) and, to a lesser extent, of the collection year (French orchard) on the community composition of the bacterial communities in fruiting bodies. In addition, since truffle fruiting bodies are likely colonized by bacteria that thrive in the surrounding soil when the embryos are formed (Antony-Babu et al., 2014), such differences could be explained by variations in the bacterial communities of the soil surrounding truffles. Soils properties and climatic conditions likely differed between the two orchards. Similarly, root microbiomes are initially strongly influenced by the composition of the communities of the bulk soil and the environmental factors that influence this "starter" community (Zarraonaindia et al., 2015; Colin et al., 2017). Local pH,

nutrients availability, or humidity levels have all been shown to significantly alter soil bacterial community composition (Uroz et al., 2016; Lladó et al., 2017). Although the general physico-chemical properties of soil are likely to be rather homogenous at the scale of a truffle orchard, it is well-known that small-scale heterogeneity exists in soil (Vos et al., 2013). We cannot exclude either that the differences between the factors influencing each orchard's microbiomes are due to divergent sampling strategies in the two sites: firstly, samples were collected over 2 and 4 years, respectively, and secondly, the two orchards differed in surface area. Altogether this indicates that a better understanding of the interactions between soil microbial communities and truffle embryo at a microscopic scale is required to foresee the process of colonization of truffle fruiting bodies by bacteria.

Taken together, our results provide an unprecedented view of the microbiome associated to the black truffle *T. aestivum*. Microbiomes dominated by either the α-Proteobacteria class, and in some cases the β-Proteobacteria or the Sphingobacteriia classes could be evidenced regardless of geographical origin. The consistent occurrence of those microbes in fruiting bodies from orchards separated by hundreds of kilometers suggests that these bacteria might be highly relevant for truffles ecology and life cycle. Our results also highlight that factors shaping truffle's microbiomes might differ based on local conditions, but unlike in other fungi, fruiting body maturation and genetic background did not seem to influence the microbiome. Overall, the findings presented here highlight the need to improve our understanding of truffle fruiting body development, of how the truffle microbiome is shaped, and what benefits it provides to truffles (or vice-versa). Complementary studies deploying large sampling efforts and functional studies of main bacterial players of the microbiome will be required to better understand these points.

AUTHOR CONTRIBUTIONS

RS designed the experiments and collected the truffle samples. AD and VM characterized the microbiome with input from SU; genotyping by SSR was done by VM, MV, MP, and SE; mating type was determined by VM, MV, MP, SE, and FP. Data was analyzed by MV, RS, AD, and JGMV. AD, JGMV, MV, and RS wrote the manuscript with input from the other co-authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01437/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplemental figures & tables to the manuscript

Orchard conditions and fruiting body characteristics drive the microbiome of the black truffle *Tuber aestivum*

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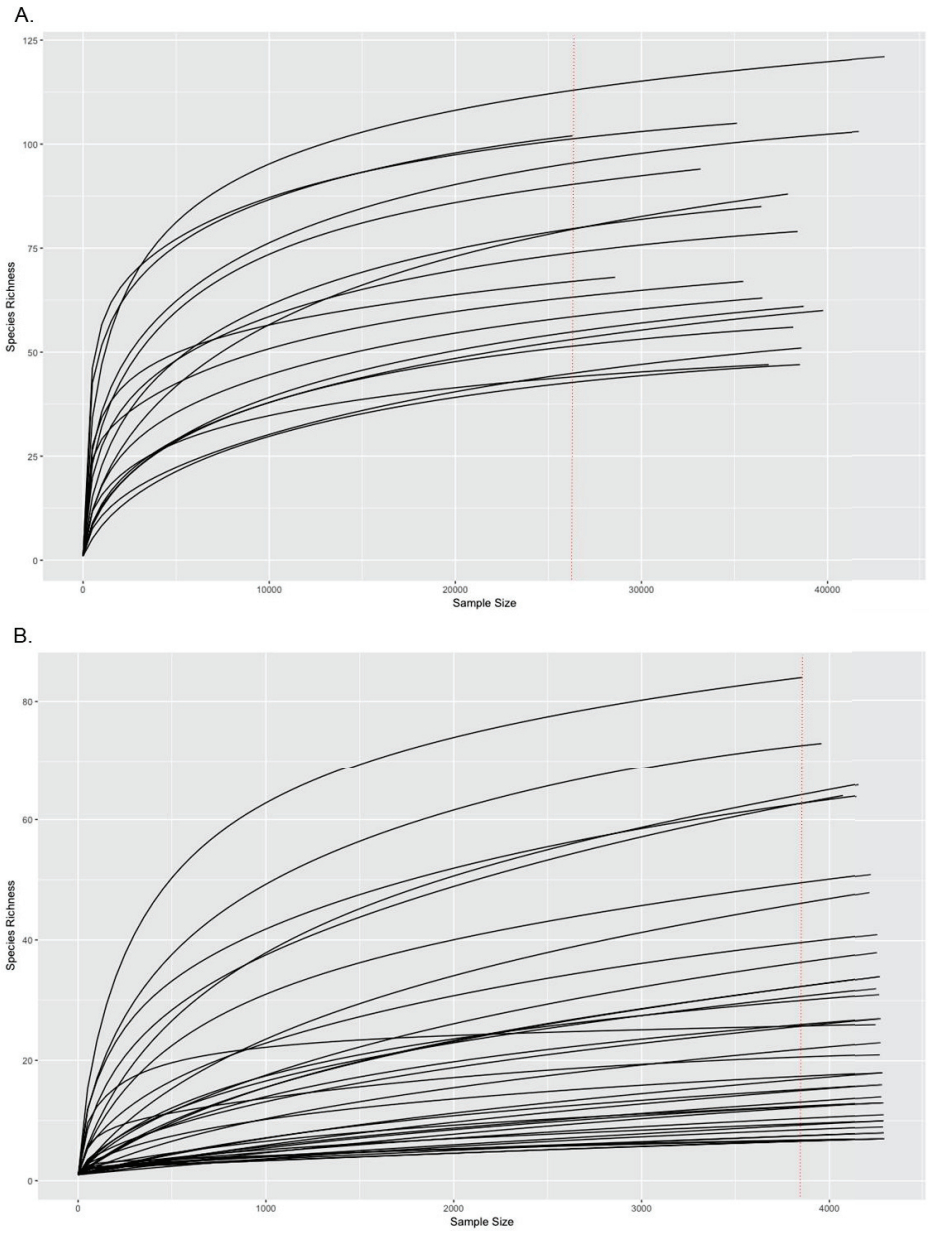
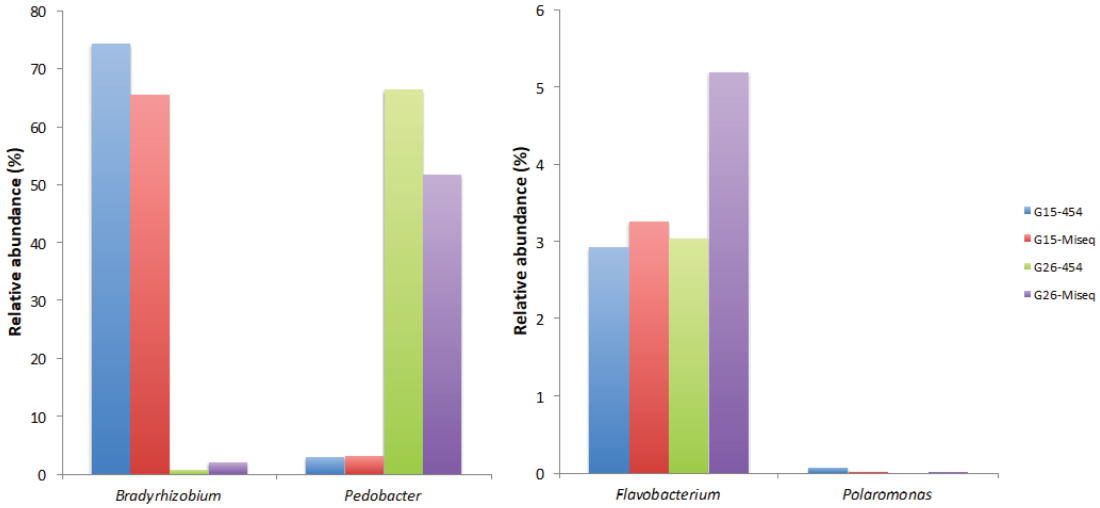


Supplemental Figure 1. Rarefaction curves
Rarefaction curves depicting OTUs numbers as a function of sequencing depth for Swiss (A, Illumina sequencing) and French truffle samples (B, 454 sequencing). The red lines indicate the value used for subsampling of the data.



Supplemental Figure 2. Microbiome of *Tuber melanosporum*
Comparison of the relative abundance of 4 important genera observed in this study as measured by 454 pyrosequecing and Illumina MiSeq of two identical samples. Total DNA of the gleba of two *T. melanosporum* truffles was extracted following the same method described here. Sequencing and data analysis was performed as described in the mat & met section.

Supplemental Table 1. List of genera commonly found in truffle ascocarps collected in the French and Swiss orchards and the average relative abundance of reads (± SE).

Genera	FR [%]	CH [%]
Bradyrhizobium	65.1 ± 8.8	58.6 ± 6.9
Pseudomonas	8.1 ± 3.4	3.4 ± 1.4
Polaromonas	5.4 ± 5.0	9.2 ± 4.4
Pedobacter	4.3 ± 3.3	13.8 ± 4.9
Flavobacterium	2.5 ± 1.2	0.81 ± 0.74
Sphingobacterium	1.1 ± 0.7	0.18 ± 0.16
Nocardioides	0.52 ± 0.20	0.19 ± 0.11
Chryseobacterium	0.43 ± 0.30	0.42 ± 0.31
Rhizobium	0.32 ± 0.12	0.98 ± 0.43
Reyranella	0.26 ± 0.24	0.020 ± 0.010
Devosia	0.21 ± 0.09	0.12 ± 0.05
Shinella	0.19 ± 0.05	0.074 ± 0.042
Mycobacterium	0.18 ± 0.07	0.082 ± 0.036
Microbacterium	0.17 ± 0.10	0.063 ± 0.036
Dyadobacter	0.16 ± 0.09	0.076 ± 0.030
Stenotrophomonas	0.11 ± 0.08	0.56 ± 0.49
Streptomyces	0.11 ± 0.06	0.099 ± 0.059
Clostridium	0.100 ± 0.075	0.060 ± 0.017
Opitutus	0.096 ± 0.058	0.059 ± 0.029
Acinetobacter	0.089 ± 0.019	0.057 ± 0.034
Kribbella	0.071 ± 0.042	0.037 ± 0.020
Luteolibacter	0.051 ± 0.025	0.070 ± 0.040
Lactococcus	0.045 ± 0.008	0.003 ± 0.002
Paenibacillus	0.033 ± 0.027	0.084 ± 0.058
Steroidobacter	0.026 ± 0.018	0.024 ± 0.012
Providencia	0.009 ± 0.004	0.001 ± 0.001
Chitinophaga	0.007 ± 0.007	0.008 ± 0.005
Mesorhizobium	0.004 ± 0.002	0.014 ± 0.008

7.4 Chapter 4: Are bacteria responsible for aroma deterioration upon storage of the black truffle *Tuber aestivum*: A microbiome and volatilome study

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(1) Concept and design

MV: 50%; RS: 50%

(2) Conducting tests and experiments

MV: 70%; AD: 30%

(3) Compilation of data sets and figures

MV: 70%; AD: 10%; RS: 20%

(4) Analysis and interpretation of data

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

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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

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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

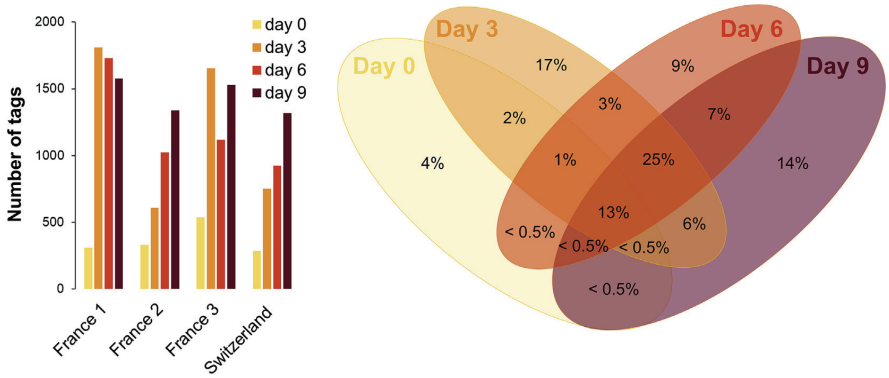

Fig. 1. Changes in the volatile profiles of *T. aestivum* fruiting bodies during storage. The bar chart illustrates the total number of volatile signals (TAGs) detected at each time point and site in at least one truffle. A strong increase in the total number of emitted volatiles (TAGs) is visible in 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

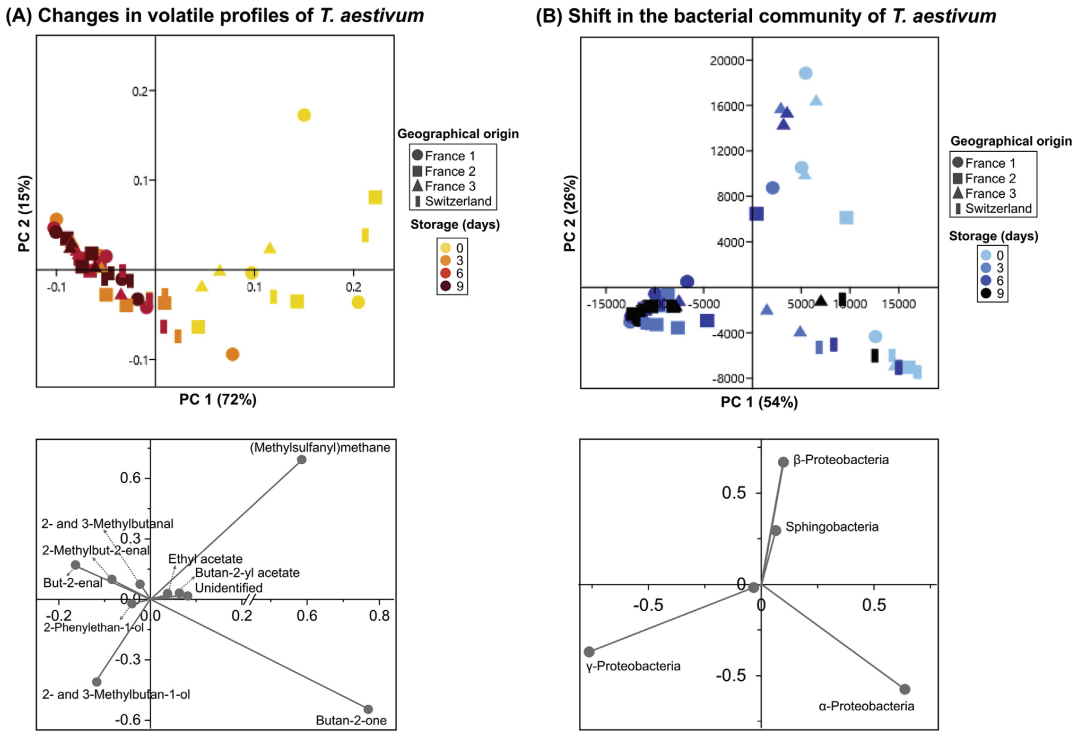

Fig. 2. Changes in volatile profiles and in the bacterial community of *T. aestivum* fruiting bodies during storage. (A) PCA based on volatiles which concentrations significantly varied during storage in truffles from at least one geographical origin ($p < 0.05$, Kruskal-Wallis test with $\alpha = 0.05$). 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.

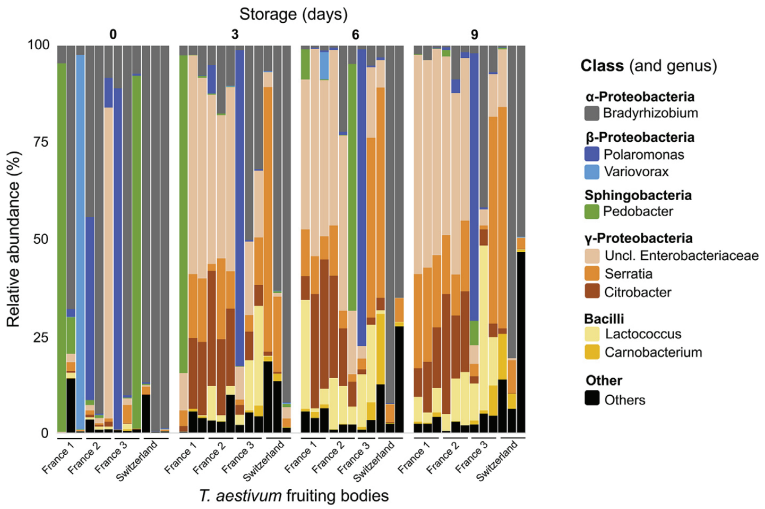

Fig. 3. Evolution of the truffle microbiome during storage. Changes in the relative proportion of the nine most abundant bacterial genera within single *T. aestivum* fruiting bodies. Each bar represents a single fruiting body.

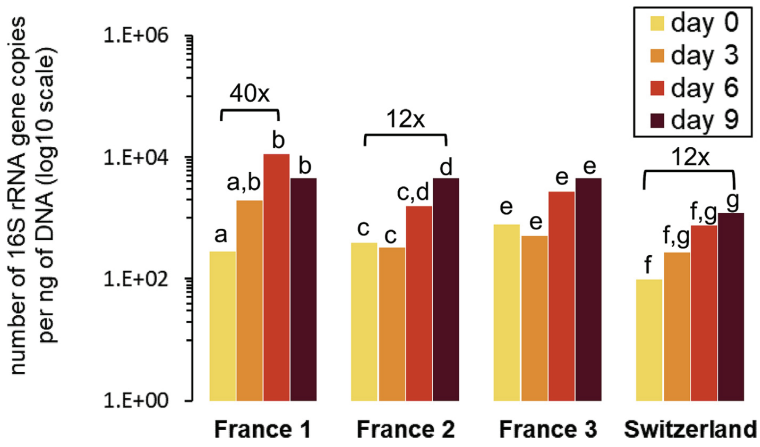

Fig. 4. Evolution of bacterial density during storage. Changes in bacterial cell density (number of 16S rRNA gene copies normalized to ng of fungal DNA) in *T. aestivum* fruiting bodies during storage. Bars represent the average values of three fruiting bodies per location and time point (days 0,3,6 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

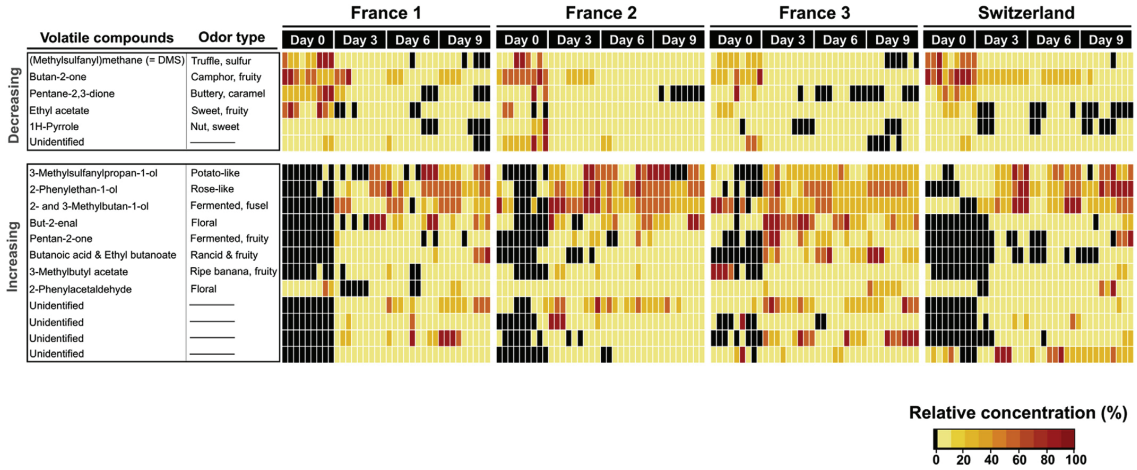

Fig. 5. Evolution of the volatile profile of truffles during storage. The heatmap illustrates volatiles consistently emitted by truffles in at least three or all the four geographical regions, and whose concentrations significantly decreased or increased with time ("freshness" and "spoilage marker",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

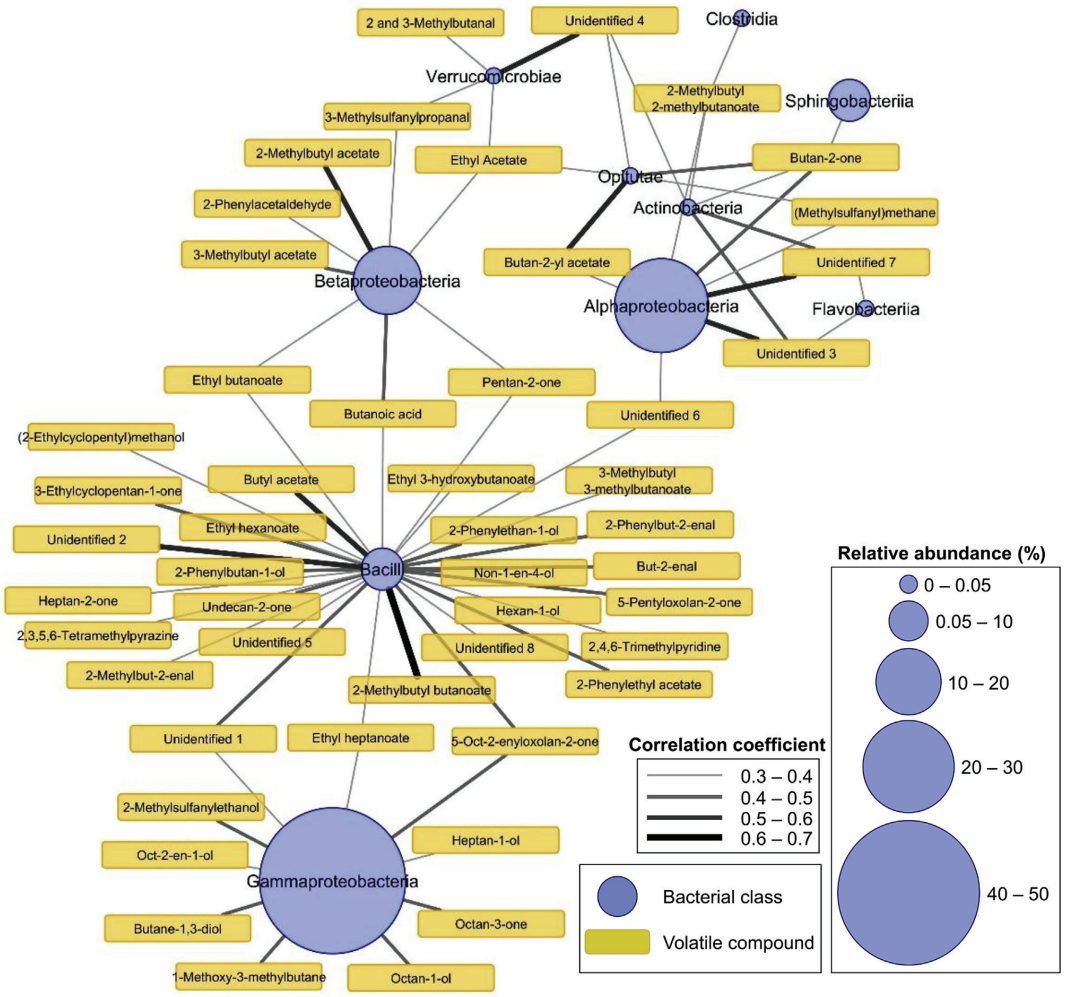

Fig. 6. Correlation network among bacteria and volatiles. Correlation between volatile compounds and bacterial communities of *T. aestivum*. Nodes represent most abundant bacterial classes and volatile compounds which significantly differed in concentration among storage times at least in one geographical 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. aestivum's 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. melanosporum*, *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.

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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.

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Appendix A. Supplementary data

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

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9 Curriculum vitae

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Thesis:	Development of test systems to evaluate the antimicrobial potential
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Thesis:	Extraction of *β*-carotene from whey protein

WORK EXPERIENCE

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PUBLICATIONS

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CONFERENCES

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