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Jun Niimi, Aurélie Deveau, Richard Splivallo

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Research

Geographical-based variations in white truffle *Tuber magnatum* aroma is explained by quantitative differences in key volatile compounds

Jun Niimi[1,4]*, Aurélie Deveau[2]* and Richard Splivallo[1,3]*

[1]Institute for Molecular Biosciences, Goethe University Frankfurt, Max-von-Laue Str. 9, Frankfurt am Main 60438, Germany; [2]Institut national de la recherche agronomique (INRA), Unité Mixte de Recherche 1136 INRA-Université de Lorraine, Interactions Arbres/Microorganismes, Centre INRA-Grand Est-Nancy, Champenoux 54280, France; [3]Nectariss Sàrl, Avenue de Senalèche 9, Pully 1009, Switzerland; [4]Present address: Division of Bioeconomy and Health, RISE Research Institutes of Sweden AB, Göteborg 412 76, Sweden

Author for correspondence:
Jun Niimi
Email: jun.niimi@ri.se

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Summary

- The factors that vary the aroma of *Tuber magnatum* fruiting bodies are poorly understood. The study determined the headspace aroma composition, sensory aroma profiles, maturity and bacterial communities from *T. magnatum* originating from Italy, Croatia, Hungary, and Serbia, and tested if truffle aroma is dependent on provenance and if fruiting body volatiles are explained by maturity and/or bacterial communities.
- Headspace volatile profiles were determined using gas chromatography–mass spectrometry–olfactometry (GC-MS-O) and aroma of fruiting body extracts were sensorially assessed. Fruiting body maturity was estimated through spore melanisation. Bacterial community was determined using 16S rRNA amplicon sequencing.
- Main odour active compounds were present in all truffles but varied in concentration. Aroma of truffle extracts were sensorially discriminated by sites. However, volatile profiles of individual fruiting bodies varied more within sites than across geographic area, while maturity level did not play a role. Bacterial communities varied highly and were partially explained by provenance. A few rare bacterial operational taxonomical units associated with a select few nonodour active volatile compounds.
- Specificities of the aroma of *T. magnatum* truffles are more likely to be linked to individual properties than provenance. Some constituents of bacteria may provide biomarkers of provenance and be linked to nonodour active volatiles.

Introduction

Truffle fungi are one of the most expensive food in the world. Hundreds of truffle species exist, but the white truffle *Tuber magnatum* is anecdotally regarded as the best and most expensive. Prices range from €3000–5000 per kg and as high as €7000 per kg (Riccioni *et al.*, 2016). *Tuber magnatum* is commonly known as the Alba or Piedmont truffle named after the Italian region where in the late middle ages truffles were already well known (Rittersma, 2011). The natural distribution of *T. magnatum* however extends throughout the Italian territory, south east France and the eastern European countries of Hungary, Croatia (Istria), Serbia, Bulgaria, Greece, Slovenia, and Romania (Marjanović *et al.*, 2015; Belfiori *et al.*, 2020). Why *T. magnatum* grow only in these particular countries remains uncertain, but these areas could have ecological conditions suitable for the growth and development of *T. magnatum* ectomycorrhizas and fruiting bodies (Bragato & Marjanović, 2016). Recent genetic studies on 36 *T. magnatum* populations encompassing more than 400 truffles have exemplified a clear genetic structure within Europe. Specifically, four genetic clusters for *T. magnatum* located in: (1) southern Italy, (2) central Italy and Istria, (3) northern Italy, and (4) the Balkan/Pannonian regions were revealed (Rubini *et al.*, 2005; Belfiori *et al.*, 2020), and further support the inference that *T. magnatum* might have recolonised Europe after the last ice age starting from central Italy (Belfiori *et al.*, 2020).

Truffles owe their reputation to their unique intense smell. More than 60 volatile compounds have been detected in *T. magnatum*, but only 11 contribute to the smell (Schmidberger & Schieberle, 2017). At its core is 2,4-dithiapentane (DTP), a sulphur-containing compound with a garlic character. This molecule was isolated and characterised from *T. magnatum* in the 1970s (Fiecchi *et al.*, 1967) and its synthetic version has since been used as food flavouring (Wernig *et al.*, 2018). Other important contributors to *T. magnatum* aroma include 3-(methylthio) propanal (potato character), 2- and 3-methylbutanal (malty character) and 2,3-butanedione (buttery character) (Schmidberger & Schieberle, 2017). A recent study reported 115 volatiles from

*All authors contributed equally to this work.

T. magnatum fruiting bodies with site-specific compounds from among 16 locations in Italy and one site in Croatia (Istria), confirming earlier reports of volatile markers exclusively occurring in specific regions (Gioacchini *et al.*, 2008; Vita *et al.*, 2018). Many of these site markers were sulphur-containing compounds or terpenes. Site markers therefore might influence truffle aroma, provided that the markers are odour active and at levels above perception threshold. Recently, volatile marker compounds that can classify *T. magnatum* truffles based on country of origin were preliminarily reported including four from Slovenia and five from Italy (Strojnik *et al.*, 2020). This raised questions that are currently unanswered: whether these compounds are exclusive to specific countries or are ubiquitous but present in varying proportions by country, the contribution of these compounds to perceptual differences in aroma, and the existence of additional marker compounds from multiple countries.

From the aforementioned studies, volatiles measured from truffles appear to vary across provenance, but the role of specific factors that might vary across provenance and in turn potentially influence white truffle aroma remain mysterious. Numerous factors have been suggested to influence truffle aroma including fruiting body maturity (Zeppa *et al.*, 2004), host tree association (Gioacchini *et al.*, 2008; Vita *et al.*, 2018), genetics (Splivallo *et al.*, 2011; Molinier *et al.*, 2015; Vahdatzadeh & Splivallo, 2018), microbes colonising truffle fruiting bodies (Splivallo *et al.*, 2015; Vahdatzadeh *et al.*, 2015), and other environmental factors (i.e. soil, climate, season). As with other plants and fungi, each of those factors could possibly influence truffle aroma, but there is currently no consensus. For instance, maturation might have an influence on the aroma of *Tuber borchii* but not on *Tuber aestivum*; for *T. magnatum* this is unknown.

Some factors have been postulated that may influence aroma, volatile profile and the microbiome of various truffle species. From this perspective three hypotheses were postulated:

(**1**) H_1 – Aromas of truffle fruiting bodies are different based on provenance

(**2**) H_2 – Volatile profile and bacterial community of individual *T. magnatum* truffle fruiting bodies are influenced by provenance and fruiting body maturity

(**3**) H_3 – Volatile profile of individual *T. magnatum* fruiting bodies are influenced by the bacterial communities.

To test these hypotheses, *T. magnatum* truffles originating from seven orchards and four countries were analysed. First, volatile compounds and therefore the aroma of truffles were determined. Second, the bacterial community of individual truffle fruiting bodies from the varying provenances were determined. Thirdly, fruiting body maturation, truffle provenance and bacterial community were analysed as potential factors that could explain aroma variability among single truffle fruiting bodies.

Materials and Methods

Chemicals

Silicon oil, 2,3-butanedione (99%), dimethyl sulfide (DMS) (≥99%), (E)-2-octenal (95%), trans-2-hexenal (96%), isovaleric acid (99%) were purchased from VWR (Darmstadt, Germany). 2,3-Pentanedione (≥ 96%), dimethyl sulfone (98%), 1-octen-3-ol (98%), 2-methyl butanal (95%), 3-methyl butanal (97%), heptanal (≥ 95%), hexanal (98%), (E,E)-2,4-nonadienal (≥ 89%), methional (≥ 97%), benzaldehyde (≥ 98%), benzeneacetaldehyde (≥ 95%), 2,4-dithiapentane (DTP) (≥ 99%), dimethyl disulfide (DMDS) (≥ 99%), dimethyl trisulfide (DMTS) (≥ 99%), nonanal (≥ 95%), 2-methyl-2-pyrroline, and alkane series standard solution (C8-C20) in hexane were purchased from Sigma-Aldrich (Taufkirchen, Germany). Short chain alkane standard mixture (C5–C8) was prepared in house: pentane, hexane, heptane (99%) (VWR), and octane (99%) (Sigma-Aldrich).

Biological material and sample processing

Truffle fruiting bodies of *T. magnatum* were collected from natural truffle orchards in four countries (Italy, Hungary Serbia and Croatia) during one truffle season (October 2018–January 2019; Table 1). Two locations were sampled per country apart from Croatia (one site) and at least four truffles were collected per site/truffle orchard by professional hunters using trained dogs (Table 1). Precise locations are not provided due to confidentiality of the truffle hunters. The harvested truffles were wrapped in paper towels and packaged at 4°C with cooler pads and immediately sent by express courier to our laboratory and received within 3 d. Truffle fruiting bodies upon arrival to the laboratory were immediately processed using the protocol explained hereafter (summarised in Fig. 1). Species identity was confirmed for every single fruiting body by spore morphology (when visible) and by PCR using *T. magnatum* species-specific primers, as published previously (Rizzello *et al.*, 2012).

Upon arrival, each truffle was cleaned with a brush under running cold water and dried with paper towels. The mass of fruiting bodies was recorded before removing the peridium with sterile knives. Multiple subsamples were taken from each fruiting body gleba to analyse maturity (75 ± 25 mg) (Fig. 1-2a), volatiles (300 ± 5 mg) (volatile analysis of individual fruiting bodies will heretofore be referred to has volatile organic compounds (VOC)) (Supporting Information Methods S1; Fig. 1-2b), and bacterial characterisation (75 ± 25 mg) (Methods S2; Fig. 1-2c). The maturity and bacterial subsamples were placed in sterile 1.5 ml Eppendorf tubes and kept at − 20°C until further processing. Truffle maturity was determined by estimating the percentage of ascii containing immature spores (Zeppa *et al.*, 2004). DNA extraction, library preparation, sequencing procedures and bioinformatics for bacterial community analyses were performed as previously described (Splivallo *et al.*, 2019) (Methods S2). Fungi were not considered in the analysis as the method employed was especially suited to bacterial characterisation of truffles. Standard procedures for characterising fungal communities using high-throughput sequencing are ineffective due to the massive amount of DNA in the truffle that prevents the detection of other fungi despite their presence (A. Deveau, unpublished). Subsamples for volatile analysis were analysed fresh, immediately after processing, using a validated method in which a single truffle subsample

Table 1 Sample information of the *Tuber magnatum* fruiting bodies investigated.

Location (region)	Sample code	Collection date	No. fruiting bodies	Mean maturity (% ± SE)	Mean weight (g ± SE)
Hungary 1 (Baranya)	HUN1	10/2018	7	81.8 ± 2.0	11.8 ± 1.2
Hungary 2 (Somogy)	HUN2	11/2018	4	74.7 ± 2.6	29.6 ± 6.6
Italy 1* (Abruzzo)	ITA1	11/2018	6	67.1 ± 5.9	31.3 ± 13.9
Italy 2** (Abruzzo)	ITA2	11/2018	5	68.8 ± 2.3	39.9 ± 3.5
Serbia 1 (Kalubara)	SER1	12/2018	6	83.9 ± 1.4	24.8 ± 2.6
Serbia 2 (Srem)	SER2	12/2018	5	90.6 ± 1.7	30.4 ± 3.3
Croatia 1 (Istria)	CRO1	01/2019	7	45.1 ± 5.0	28.3 ± 7.2

* and **, ITA1 and ITA2 correspond to two natural but distinct truffle orchards located in Abruzzo region.

is sufficient to properly represent the volatilome of each truffle (Splivallo *et al.*, 2012).

The remaining fruiting body gleba from a single site were grated with an electric grater (WMF, Geislingen) for pooling truffles to represent that site. The fruiting bodies in experimental design Fig. 1-1 were pooled to have sufficient amount of sample to perform GC-O (Fig. 1-1a) and sensory analysis (1-1c). It also served as a compromise for practicality, when subjecting 40 fruiting bodies for sensory evaluation to each panellist is too many for assessment, time intensive and therefore impractical. Furthermore, a sample preparation method that allows all samples to be assessed at once for sensory analysis was required, and waiting until all truffles arrived was not an option due to their short shelf-life. The grated truffles from a given site were mixed with a sterile spoon and sampled into 11 solid phase microextraction (SPME) vials (300 ± 5 mg) in preparation for gas chromatography–olfactometry (GC-O) (total eight vials per sample) (Fig. 1-1a) and volatile analysis (GC-mass spectrometry (MS)) (total of three vials per sample) (Fig. 1-1b) (Methods S1). Grated fruiting bodies in SPME vials were held at 5°C overnight until olfactometry the following day. The remainder of the grated fruiting bodies were homogenised with silicon oil (50 g truffle: 100 g silicon oil ratio) (Ultra-Turrax®; IKA, Staufen, Germany) and centrifuged in 30 ml centrifuge tubes at 8000 ***g*** for 10 min at 5°C (Heraeus

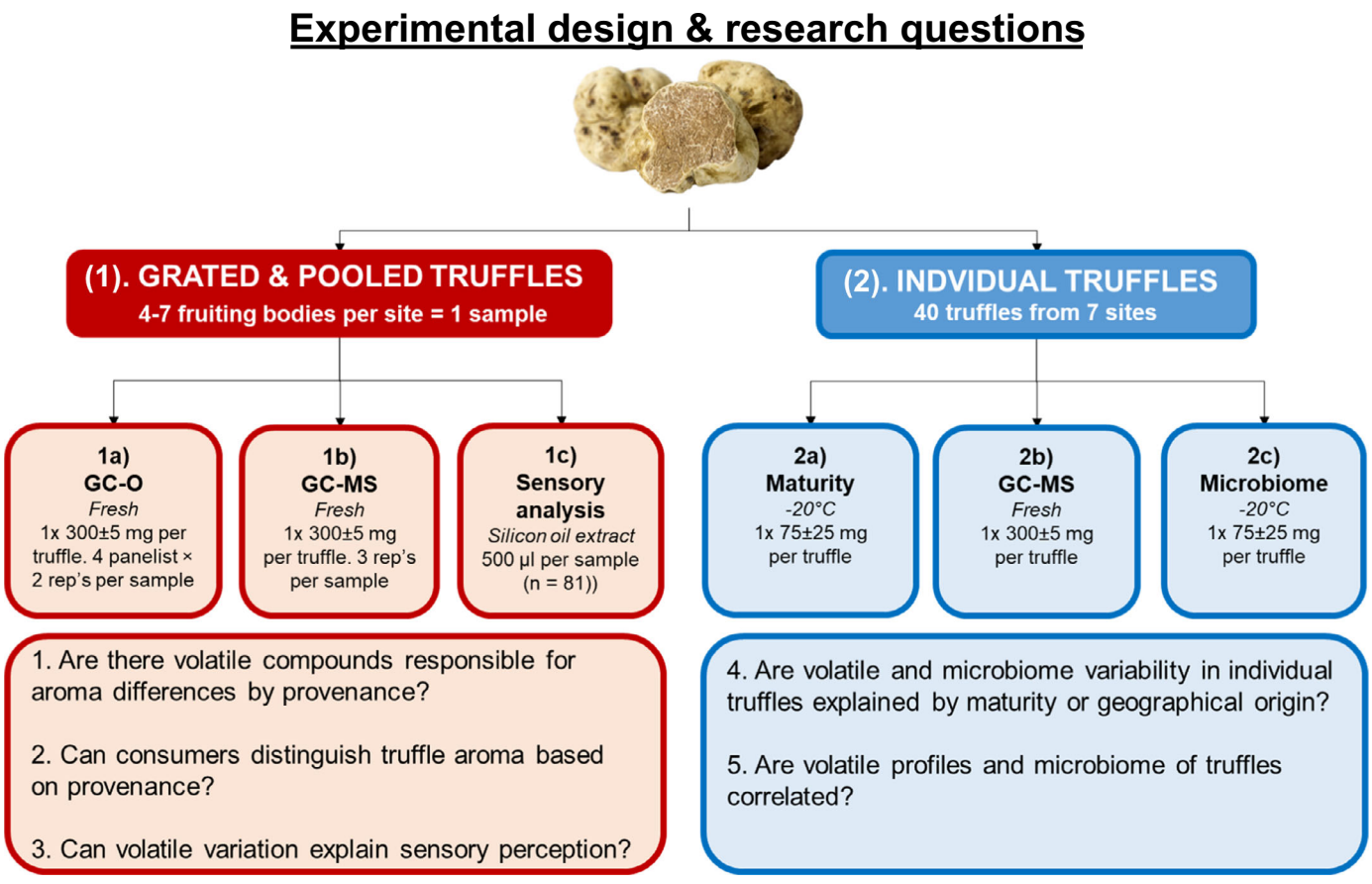

Fig. 1 Experimental design for the analysis of individual and pooled *Tuber magnatum* truffles and key questions associated with each design.

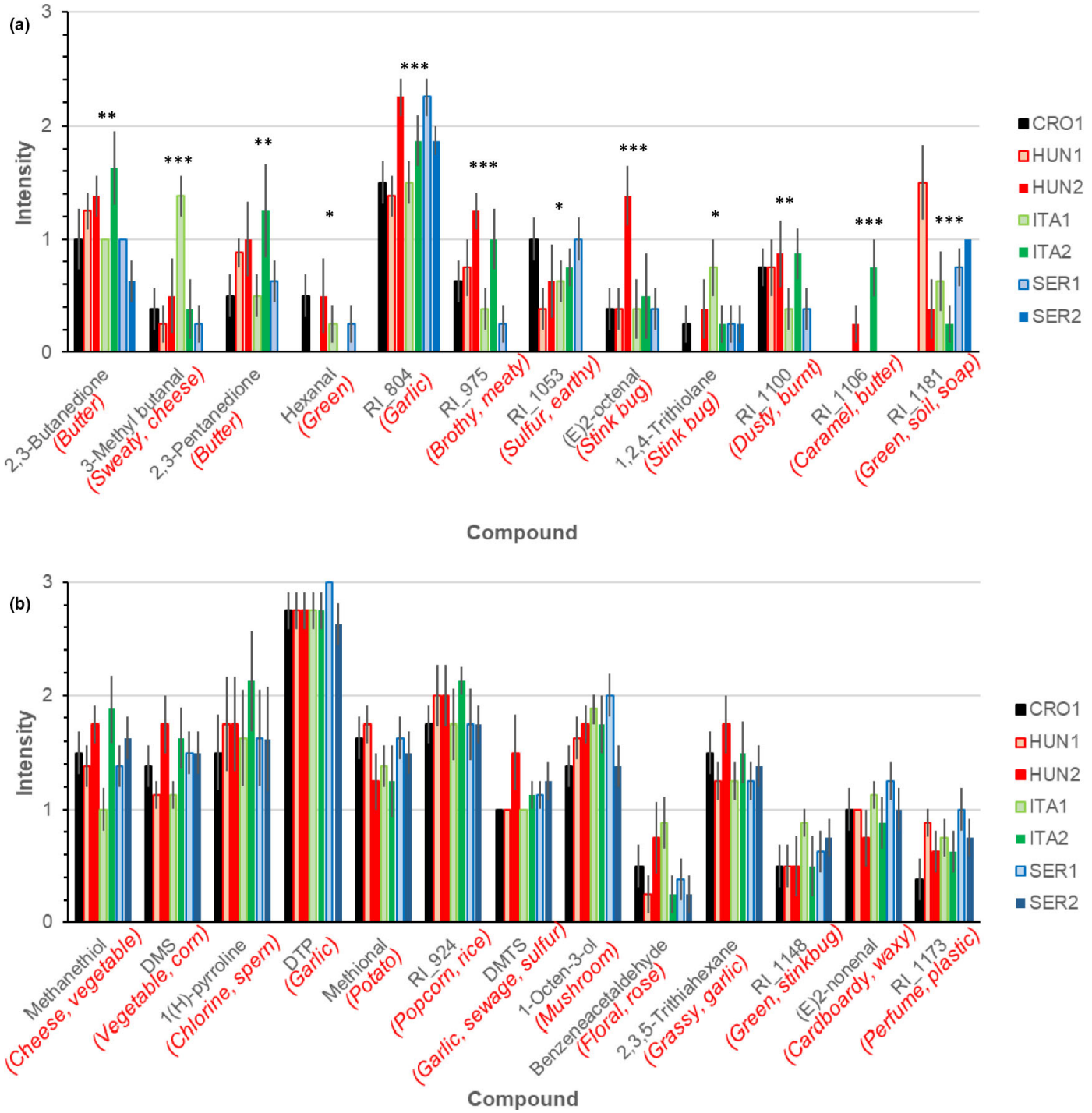

Fig. 2 Mean odour intensities (±SE) measured from gas chromatography–olfactometry (GC-O) of ubiquitous compounds identified in *Tuber magnatum* fruiting bodies (pooled) with accompanying odour descriptions in parentheses ($n = 4$ panellists). (a) Compounds that significantly differed in intensity across sample sites and (b) compounds that did not significantly differ across samples. Compounds with numbers are retention indices (RI_) calculated through HP5-MS. Statistics according to univariate ANOVA; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Megafuge 8R centrifuge; Thermo Fisher Scientific, Osterode am Harz, Germany). The oil supernatant was decanted into 40 ml amber vials with Teflon lined caps and stored at −20°C until sensory evaluation (Fig. 1-1c). Silicon oil was used as the extraction medium due to their neutral aroma and their ability to extract aroma that gave the most similar aroma character to fresh fruiting bodies, without using chemical solvents. The aroma of truffle extracts was sensorially evaluated by consumers ($n = 81$) using the rate all that apply (RATA) method. The method consists of presenting a list of attributes relevant to the truffle aroma,

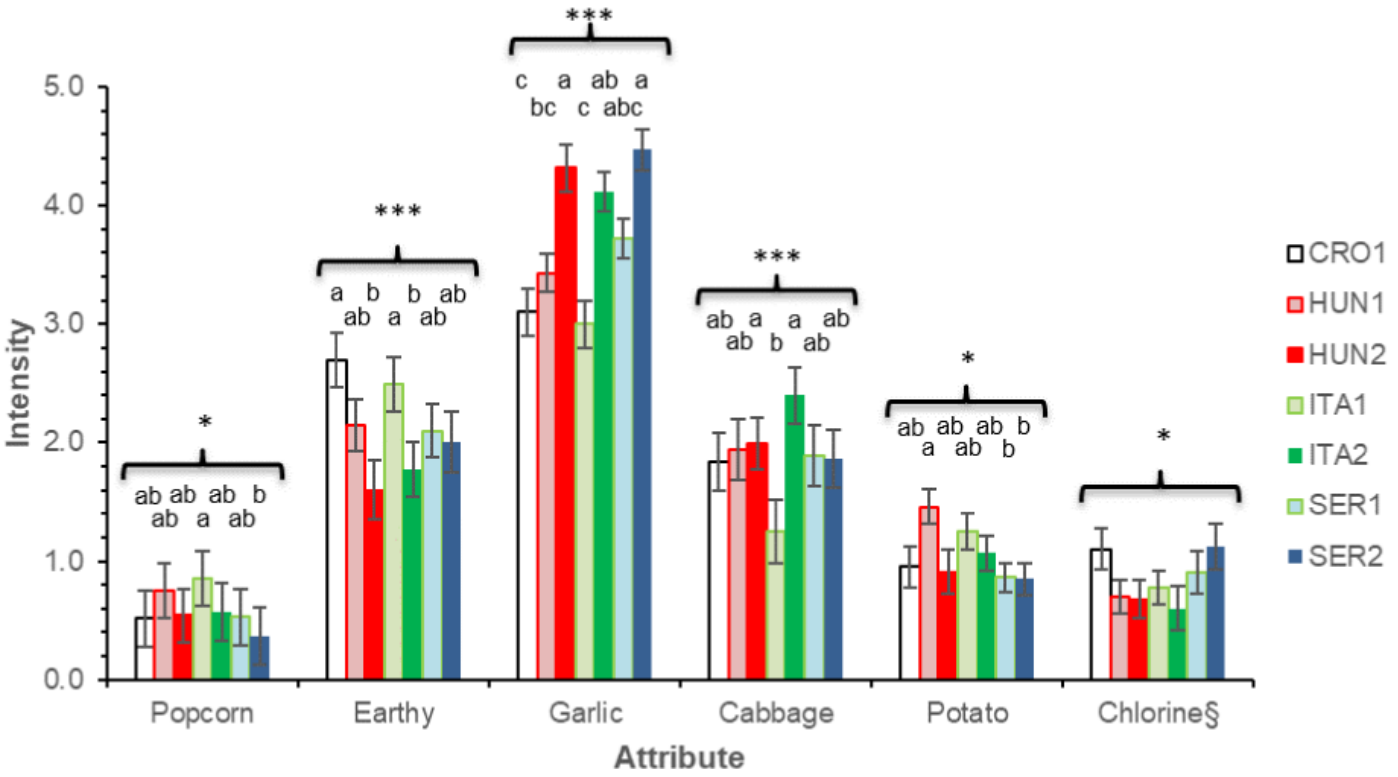

Fig. 3 Mean intensities (± SE) of significantly different rate all that apply (RATA) attributes ($n = 81$) of *Tuber magnatum* aroma extracts. ITA, Italy; HUN, Hungary; SER, Serbia; and CRO, Croatia. Statistical analysis is according to univariate ANOVA; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Means with the same superscript per attribute are not significantly different according to Tukey's honest significant difference (HSD) post hoc test. §Despite 'chlorine' being a significantly different attribute by sample, Tukey's HSD could not separate the means.

the consumers smelling the extracts and rating on categorical scales the intensity of aroma attributes that apply to the aroma characteristic of each sample (for further details, see Methods S3 and Table S1). The consumers were 57% female and 43% male. Most of the participants belonged to 18–24 and 25–34 age groups (45.7% each), while that of 35–44 and 55–64 were minor (6% and 3%, respectively). Most consumers (74%, $n = 81$) were familiar with or had previous exposure to truffle products.

Data analysis

The GC-MS data were imported as CDF files, and deconvoluted with PARADISE (v.3; Copenhagen) (Johnsen *et al.*, 2017). The resultant extracted compounds were integrated and calculated for their retention index (RI) based on an alkane series (C5–C8 and C8–C20) (van Den Dool & Kratz, 1963). Compounds were identified through the NIST 2017 GC-MS database (NIST, MD, USA), pure standards or from the literature for fragmentation patterns. The peaks of chromatograms from VOCs were normalised by dividing individual peaks by the total ion count (TIC) and analysed using principal component analysis (PCA) and UNSCRAMBLER (v.10; Camo, Oslo). The chromatogram peaks from the pooled truffle fruiting bodies (measured as with the GC-O samples) were processed in a similar manner as described above, only that the odour active compounds (OAC) identified (based on GC-O) were quantified through calibration curves. The quantified OAC were subsequently analysed using one-way ANOVA and SPSS statistics v.25 (SPSS Inc., Chicago, IL, USA) and with Fisher's least significant difference (LSD) post hoc test.

GC-O data were manually aligned across all assessors for both replicates per sample. OAC undetected by certain panellists were replaced with 0 scores. The entire data matrix was analysed using univariate ANOVA taking samples and panellist as fixed and random effects, respectively. Data from RATA (sensory evaluation) were first preprocessed by replacing missing values with 0 scores followed by univariate ANOVA, taking samples and consumers as fixed and random effects, respectively and Tukey's honest significant difference (HSD) post hoc test. Fruiting body maturity and masses were analysed with descriptive statistics and one-way ANOVA.

Details of bioinformatics processing of bacterial amplicon sequencing data are given in Methods S2. In brief, obtained sequences from amplicon sequencing were analysed using FROGS (Find Rapidly operational taxanomic unit (OTU) with Galaxy Solution) (Escudié *et al.*, 2017) following standard operating procedures. Rare OTUs (≤0.005% of all sequences in all samples) were excluded for further analyses. Clusters were affiliated to one taxonomy by blasting OTUs against the SILVA database (Quast *et al.*, 2012). OTUs were rarefied (adjusting sequences randomly to the total abundance in the smallest sample) to 45 846 using the PHYLOSEQ package in R (McMurdie & Holmes, 2013). The raw data were deposited in the NCBI Sequence Read Archive website (http://www.ncbi.nlm.nih.gov/sra) under the BioProject study accession no. PRJNA663751.

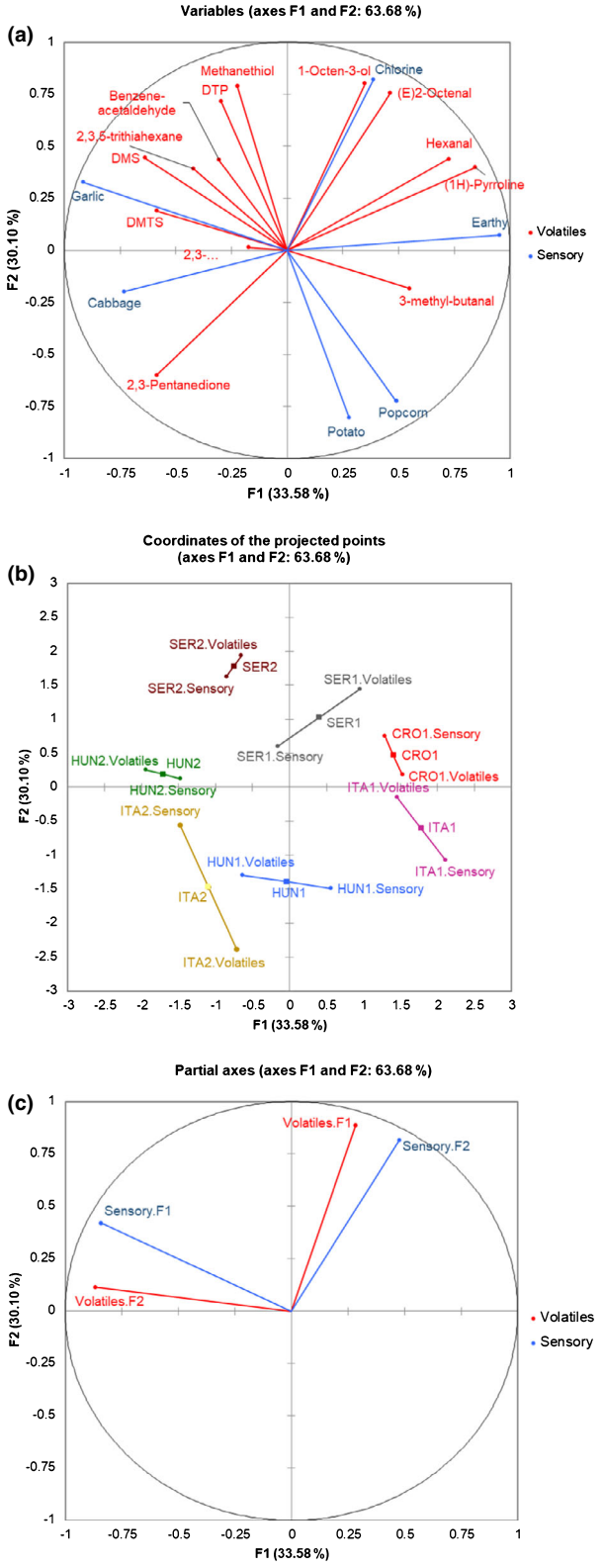

Fig. 4 MFA plots exploring the correlations between odour active volatile compounds of pooled *Tuber magnatum* fruiting bodies by site and the sensory properties determined by rate all that apply (RATA) of the truffle fruiting body extracts. (a) Loadings, (b) scores, and (c) partial axes of the two data sets for the first two factors. The overall explained variances of the first two factors accounted for 63.7% of the variation of both volatiles and sensory data together. The RV coefficient determined between the two data sets were acceptable at 0.631. The direction of the partial axes for the first two components of the volatiles and sensory data sets showed good agreement in the discrimination of scores.

Statistical analyses and data representations were performed using R-statistic (73, RSTUDIO v.1.2.5001). Differences between bacterial community structures of fruiting bodies collected from different sites were tested using permutational multivariate analysis of variance (pairwise PERMANOVA) based on Bray–Curtis distances and differences in structures were visualised using principal coordinates analysis (PCoA) and Bray–Curtis dissimilarity matrix. Fisher test followed using a Benjamini and Hochberg correction (false discovery rate correction) was used to detect significant differences in the relative abundance of bacterial OTUs between sites. Venn diagrams were produced from binarised dataset using the LIMMA package.

The quantified volatile profiles from pooled truffle samples were correlated with the significantly different attributes ($P < 0.05$) of the RATA data using multiple factor analysis (MFA) (XLSTAT v.2020; Addinsoft, New York, NY, USA). Correlation of the data projections was determined with RV coefficients.

The VOCs were correlated with the bacterial data using regularised canonical correlation analysis (rCCA) through the MIXOMICS R package (Rohart *et al.*, 2017). Normalised to TIC volatile data were correlated against the rarefied reads of OTUs bacterial data (found in at least three fruiting bodies).

Results

Truffle odorants are ubiquitous among sites but vary in concentrations

The headspace of fresh truffle fruiting bodies (grated and pooled by sites; Fig. 1-1a) were determined for OAC by GC-O and 25 compounds were olfactorily detected (Fig. 2). All compounds (except hexanal, 1,2,4-trithiolane, and compound RI1106) were ubiquitous across all samples; few unique compounds were present within a single site. However, 12 compounds (a total of 48% of compounds) significantly differed in perceived odour intensities by sites ($P < 0.05$, Fig. 2a). The intensities of the 13 remaining compounds were similar across the samples, suggesting ubiquity (Fig. 2b). The three compounds that were not olfactorily ubiquitous did not exclude the possibility that they were present in all samples, but possibly below olfactory detection thresholds. Furthermore, none of them was unique to any single site or region.

Concentrations of volatile compounds detected through GC-MS in the headspace of pooled and grated truffle samples were determined for 13 OAC (Fig. 1-1b), of which 12 significantly differed ($P < 0.05$) in concentration across sites (Table 2). ITA1 had consistently the highest concentration of 3-methyl butanal. DTP was detected in general at high concentrations, with SER2

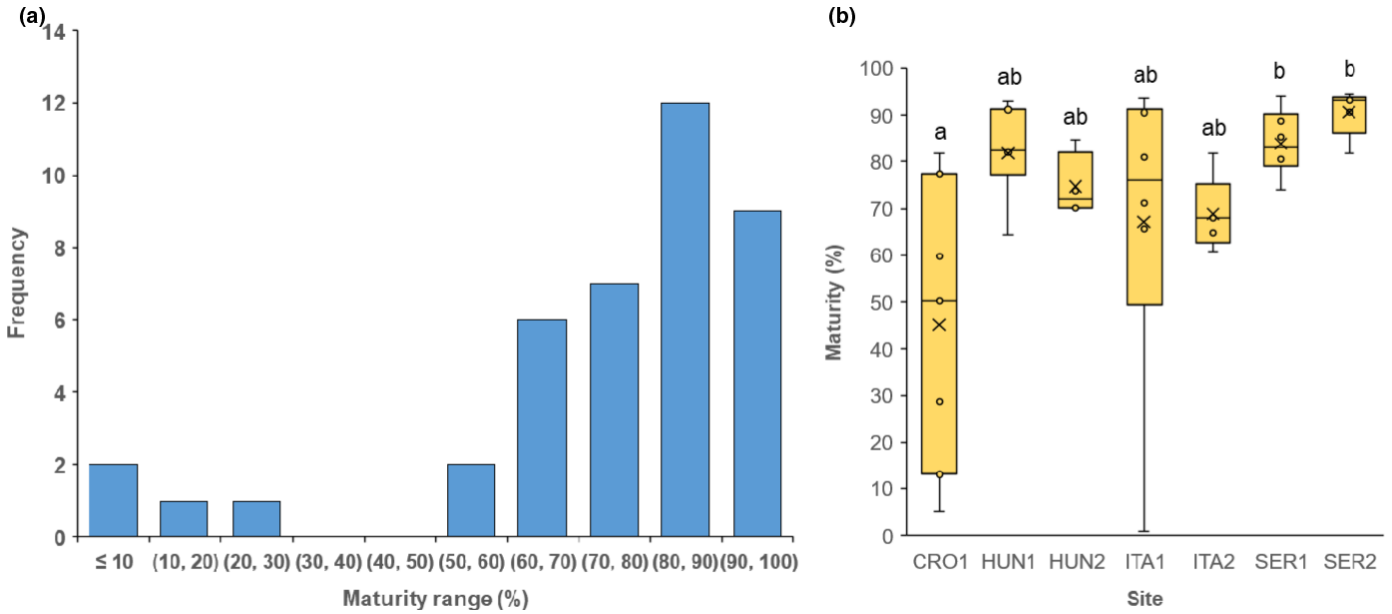

Fig. 5 *Tuber magnatum* truffle fruiting body maturities. (a) distribution of fruiting body maturities and (b) box and whisker plots of individual fruiting body maturities from seven sites showing the extreme samples, upper and lower quartiles, the mean (x), median, and individual fruiting bodies (circles). Superscripts above the box and whiskers of maturities by region denote for significantly different means using Tukey's honest significant different post hoc test.

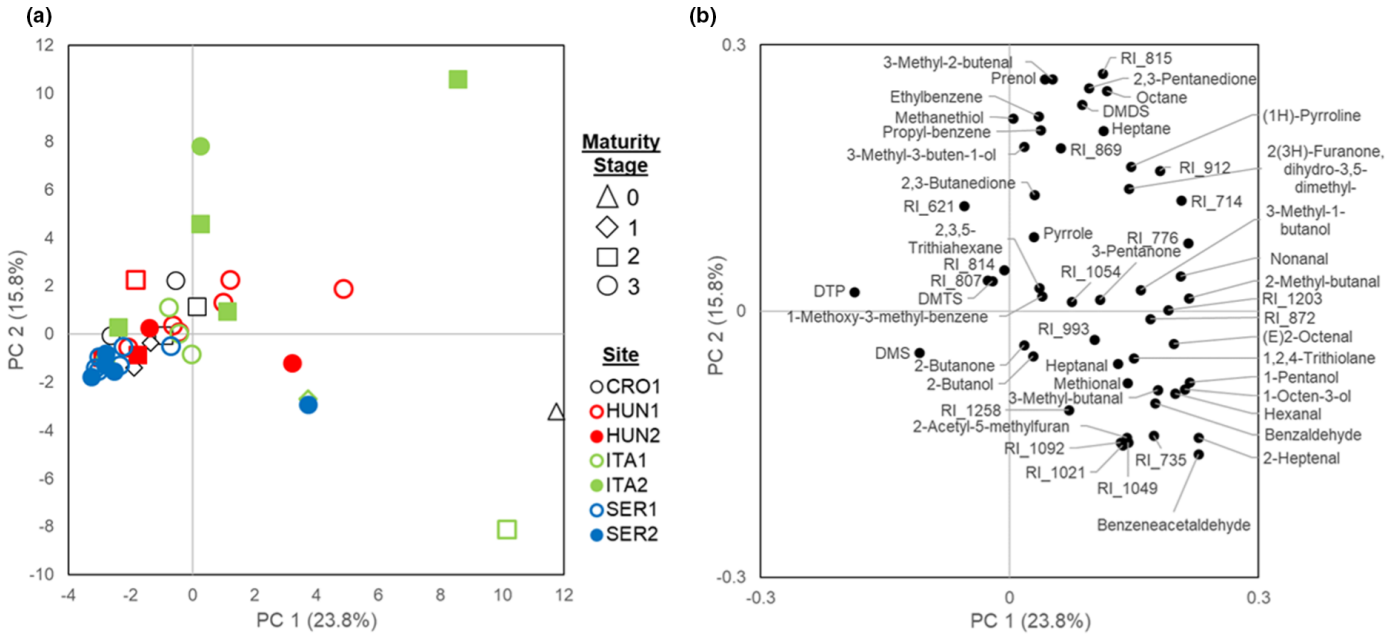

Fig. 6 Principal component analysis (PCA) scores (a) and loadings (b) plots of individual *Tuber magnatum* fruiting bodies and significantly different ($P < 0.05$) volatile compounds. DMS, dimethyl sulfide; DMDS, dimethyl disulfide; DMTS, dimethyl trisulfide; DTP, 2,4-dithiapentane; RI, retention index; denotes for unidentified. Fruiting body maturities were categorised as percentage of asci- ; stages 0 (0–5%), 1 (6–30%), 2 (31–70%) and 3 (>70%) (Zeppa *et al*., 2004).

being the highest. Odour intensity of DTP from GC-O was very high across all sites and was not significantly different; the limited scale resolution may have influenced perceptual discrimination. Six compounds were significantly different in concentration but not in perceived intensities across sites: methanethiol, DMS, (1H)-pyrolline, DMTS, 1-octen-3-ol and benzeneacetaldehyde. These compounds perhaps did not exceed the minimum concentration difference required for perceptually noticeable differences (difference threshold). Finally, 2,3,5-trithiahexane concentrations were not significantly different across sites. This was reflected by

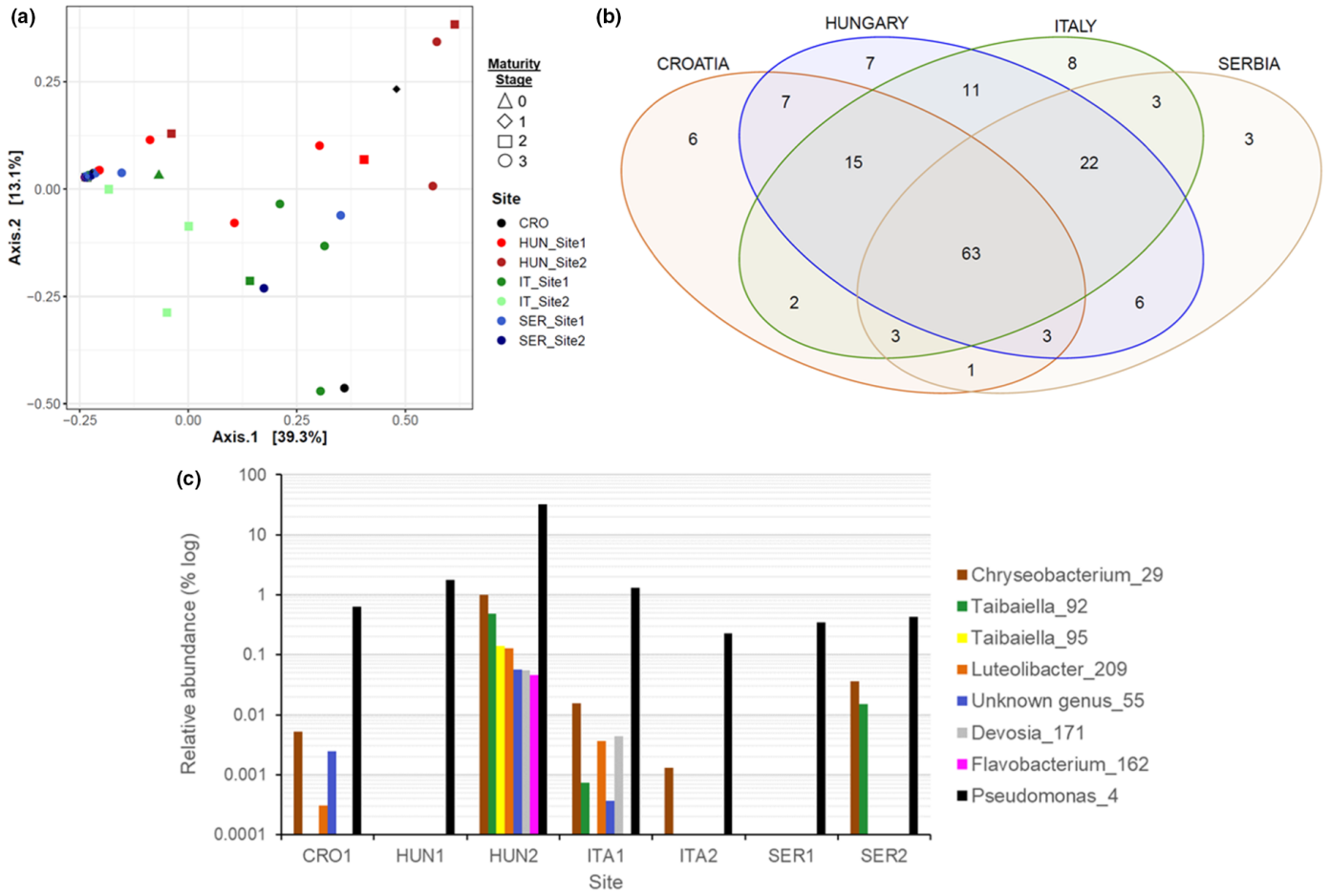

Fig. 7 Effect of geographical origin on *Tuber magnatum* microbiome. (a) Principal coordinates analysis (PCoA) representation of fruiting body microbial communities across sites and biogeographic area based on Bray–Curtis dissimilarity matrix, (b) Venn diagram, and (c) Distribution of average relative abundance of operational taxanomic units (OTUs) significantly enriched in Hungary site 2 compared with other sites (log scale).

the high intensities detected by the assessors through the olfactometry in all samples (Fig. 2). The combination of GC-MS of pooled truffle fruiting body data together with GC-O showed the necessity for both data for accurate inferences in possible variations of *T. magnatum* aroma profiles.

Consumers can discriminate representative aroma of *T. magnatum* by site

In a second step, we separately recruited consumers ($n = 81$) to profile the aroma of truffle extracts of differing provenance. Six out of 14 attributes significantly differed (univariate ANOVA; $P < 0.05$) (Fig. 3). HUN2 and SER2 samples were significantly more intense in garlic aroma than the other samples. CRO1 was characterised by an earthy and chlorine character, while HUN1 was significantly more intense in potato and popcorn. ITA2 showed the highest intensity in cabbage aroma, while ITA1 was more intense in popcorn, although the overall range of intensities for popcorn was low. The sample SER1 did not discriminate strongly by any attribute. Consumers could discriminate the aroma of samples despite the truffles being presented as extracts. The provenance of truffles could be discerned by site at a global level through these six attributes.

Correlation between the volatile profiles and the sensory characteristics perceived by consumers

Single volatile compounds can have an odour character of their own but can contribute differently in a mixture of compounds. To determine the compounds responsible for the perceived differences in global truffle aromas across sites, the quantified volatile data (GC-MS of pooled fruiting bodies) were correlated with the sensory data (RATA) using MFA (Fig. 4). Sensory attributes were overall projected with similar vectors as the compounds responsible: garlic with 2,3,5-trithiahexane, DTP, and DMTS, and chlorine with (1H)-pyrroline (Fig. 4a). 3-Methylbutanal singly was perceived as sweaty and cheese from GC-O, however it may have contributed to earthy, popcorn, or potato characters in compound mixtures. Similarly, the sulphur compounds may have elicited the cabbage characteristic, perceived by the RATA panellists. Nevertheless, volatiles (GC-MS) and sensory data (RATA) were in agreement with an RV coefficient of 0.631 (Fig. 4b), indicating that both methods provided a good proxy of aroma variability at the pooled truffle fruiting body level. The largest disparity was seen with the ITA2 sample, for which this was mainly driven by a combination of comparatively higher concentrations of 2,3-pentanedione and lower concentrations of hexanal, DTP, and

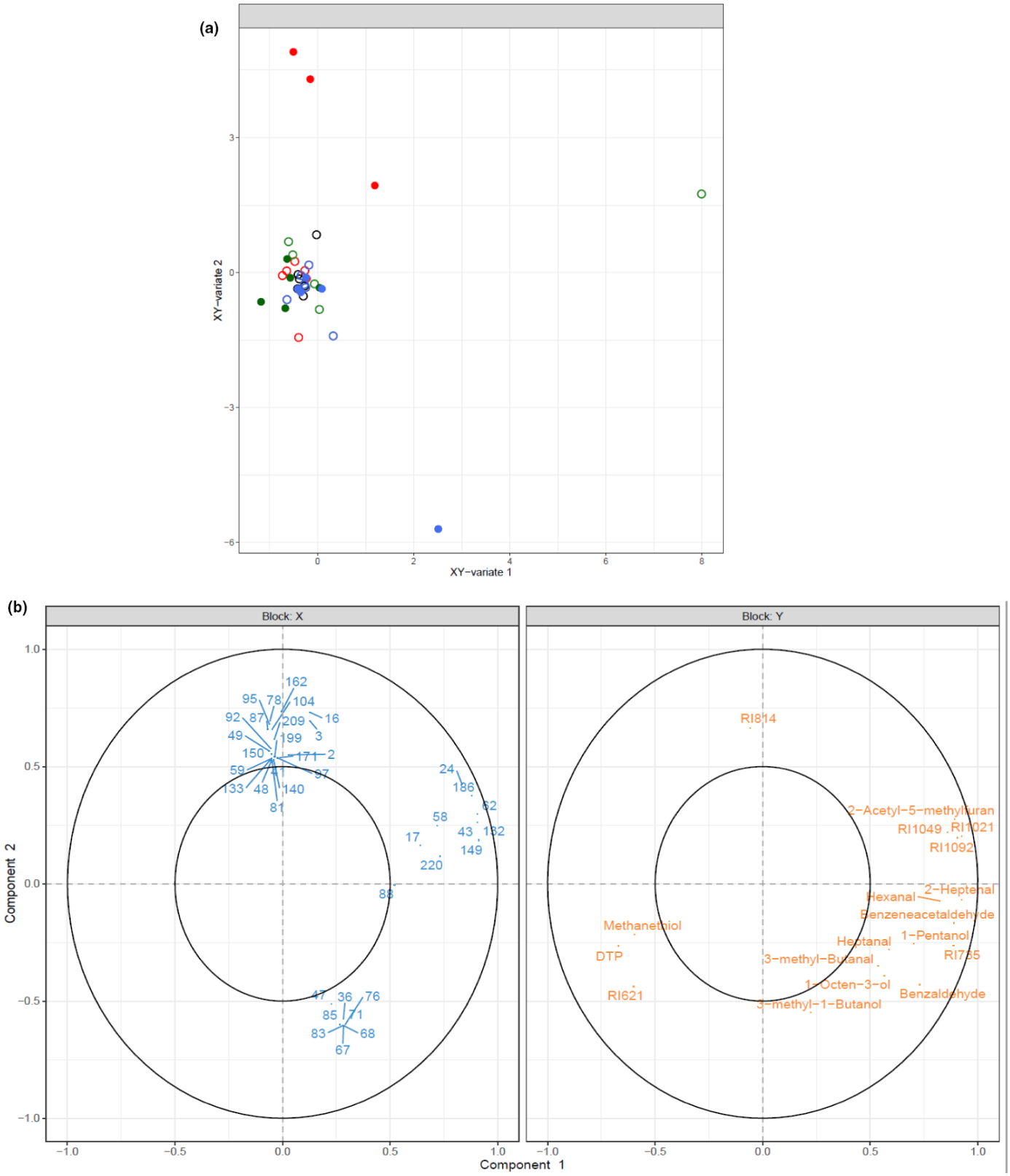

Fig. 8 Analysis of volatile and microbiome data of individual *Tuber magnatum* fruiting bodies using regularised canonical correlation analysis (rCCA). (a) Consensus fruiting body scores of microbiome and volatile profiles according to the first two variates. Correlation plot of loadings for (b) bacterial OTUs and (c) volatile compounds with covariance values > 0.5.

Table 2 Mean concentrations (μg kg) (±SE) of odour active volatile compounds from the headspace of seven fruiting body samples of *Tuber magnatum*, pooled together as sites.

Sample	RI*	CRO1	HUN1	HUN2	ITA1	ITA2	SER1	SER2	ID	*P*-value
Methanethiol[§]	< 500	1949[b] (± 169)	1373[a] (± 279)	1390[ab] (± 206)	936[a] (± 136)	1408[ab] (± 45)	2403[b] (± 207)	5223[b] (± 191)	MS, Std, RI, O	<0.001
DMS	531	25 279[a] (± 11 992)	104 618[ab] (± 48 838)	187 153[b] (± 19 644)	62 706[a] (± 34 672)	38 373[a] (± 9501)	96 921[ab] (± 4934)	164 046[b] (± 39 202)	MS, Std, RI, O	0.020
2,3-Butanedione	600	10.0[b] (± 0.2)	7.0[b] (± 1.4)	8.8[b] (± 2.6)	1.7[a] (± 0.6)	4.4[ab] (± 0.6)	4.5[ab] (± 1.0)	3.7[ab] (± 0.2)	MS, Std, RI, O	0.005
3-Methylbutanal	653	290[a] (± 13)	381[a] (± 13)	383[a] (± 42)	1082[b] (± 94)	334[a] (± 16)	395[a] (± 58)	390[a] (± 34)	MS, Std, RI, O	<0.001
(1H)-Pyrroline[‡]	685	96[b] (± 10)	56[ab] (± 1)	44[a] (± 0)	74[b] (± 13)	44[a] (± 2)	68[ab] (± 22)	66[ab] (± 10)	MS[†], RI, O	0.013
2,3-Pentanedione	701	28[b] (± 2)	29[b] (± 3)	43[c] (± 4)	14[a] (± 2)	50[c] (± 6)	13[a] (± 3)	12[a] (± 3)	MS, Std, RI, O	<0.001
Hexanal	802	366[d] (± 9)	46[ab] (± 2)	168[c] (± 87)	344[d] (± 24)	29[a] (± 4)	395[d] (± 31)	117[bc] (± 8)	MS, Std, RI, O	<0.001
DTP	892	19 256[a] (± 1467)	24 102[a] (± 8367)	32 197[a] (± 2938)	19 995[a] (± 4668)	17 751[a] (± 1206)	38 423[a] (± 1160)	147 327[b] (± 17 773)	MS, Std, RI, O	<0.001
DMTS	968	274[a] (± 4)	277[a] (± 2)	397[b] (± 65)	279[a] (± 7)	271[a] (± 1)	290[a] (± 5)	288[a] (± 3)	MS, Std, RI, O	0.037
1-Octen-3-ol	980	4.33[c] (± 0.07)	2.42[a] (± 0.04)	3.36[b] (± 0.43)	3.34[b] (± 0.03)	2.21[a] (± 0.02)	5.34[d] (± 0.42)	3.80[bc] (± 0.13)	MS, Std, RI, O	<0.001
Benzeneacetaldehyde	1045	1.62[a] (± 0.06)	4.44[b] (± 0.11)	5.89[b] (± 2.06)	4.70[b] (± 0.51)	1.84[a] (± 0.38)	4.57[b] (± 0.52)	5.97[b] (± 0.21)	MS, Std, RI, O	0.005
(E)2-Octenal	1057	5.73[c] (± 0.06)	4.82[ab] (± 0.02)	5.35[bc] (± 0.42)	5.54[c] (± 0.18)	4.70[a] (± 0.002)	5.89[c] (± 0.14)	5.35[bc] (± 0.12)	MS, Std, RI, O	0.004
2,3,5-Trithiahexane[ψ]	1124	48 (± 4)	47 (± 11)	211 (± 88)	93 (± 30)	31 (± 1)	99 (± 10)	99 (± 14)	MS, RI, O	0.059

Statistical analysis was performed using one-way ANOVA and means with the same superscript are not significantly different according to Fisher's LSD.
*RI calculated using HP5-MS.
[§]Quantified as equivalent of dimethyl sulfide.
[‡]Quantified as equivalent of 2-methyl-1-pyrroline.
[†]MS fragmentation pattern based on literature (Schmidberger & Schieberle, 2017).
[ψ]Quantified as equivalent of 2,4-dithiapentane.

2,3,5-trithiahexane (Fig. 4c). The projected scores from volatiles and sensory data of the remaining samples were closely aligned.

Fruiting body maturities varied across provenance

Truffle fruiting body maturities were determined and ranged from 1–94.5%. Out of 40 samples, 23 were highly mature (> 76% maturity), 12 were mature (51–75%), two were partially immature (26–50%) and three were immature (<25%) (Fig. 5a). Levels of maturity significantly differed across provenance (one-way ANOVA, $P = 0.006$) (Fig. 5b), when overall, fruiting bodies from SER1 and 2 had the highest proportion of mature spores, while CRO1 fruiting bodies were the least mature. Maturity variations were large within CRO1 and ITA1. Fruiting body masses ranged from 7.3 to 99.3 g (Fig. S1). Analysis with Pearson correlation showed no significant correlation between fruiting body maturity and mass, the two measures were therefore independent.

Volatile profiles of individual fruiting bodies are not explained by maturity or provenance

In the next step, we aimed to identify factors that could potentially explain variability in volatile profiles among truffles, in particular provenance and maturity. The GC-MS headspace VOCs were measured and 53 compounds were detected (Table S2). PCA of the VOCs did not highlight a discrimination of fruiting bodies based on maturity nor provenance (Fig. 6). PERMANOVA analysis failed to show that site and maturity were significant factors on the total variability of VOCs (Bray–Curtis distance, $P = 0.411$ and $P = 0.159$, respectively). Site × maturity interaction was also not significant (Bray–Curtis distance, $P = 0.102$). Instead, high variations of profiles were observed between fruiting bodies collected from the same site and same maturity. SER1 and SER2 samples were exceptions, for variation was minimal compared with fruiting bodies from the other sites. In comparing the average standard deviations across all 53 VOCs for each site (CRO1 (0.0029), HUN1 (0.0029), HUN2 (0.0057), ITA1 (0.0139), ITA2 (0.0098), SER1 (0.0009), and SER2 (0.001)), ITA samples showed the largest variation across the individual fruiting bodies. Three fruiting bodies (ITA1-FB1, CRO1-FB3 and ITA2-FB1) showed peculiar volatile profiles and drove most of the variability explained by PC1 (Fig. 6).

Despite the overall lack of discrimination between sites based on VOCs, the TIC of 19 VOCs significantly varied across sites (one-way ANOVA, $P < 0.05$), of which four were OACs detected

through GC-O and 15 were odourless/unperceivable (Fig. S2). 2-Butanol was only present in ITA1 (Fig. S3), and ethyl benzene and propyl-benzene were barely detected in SER samples. VOCs that were distinctly present/absent in specific sites or countries were rare, suggesting that most compounds even at the fruiting body level were ubiquitous, consistent with the GC-O data.

Bacterial communities vary among fruiting bodies and depending on truffle origin

In total, 2 629 495 raw reads were obtained, among which 2 550 657 were retained after bioinformatic pretreatment for further analyses (see Methods S2 for details). Numbers of reads per sample varied between 45 846 and 83 155 and were further rarefied to 45 846 in all samples. In total, 158 OTUs were detected after rarefaction among all fruiting bodies. Richness and diversity strongly varied between samples: 19 to 71 OTUs were found per fruiting body (Fig. S4a). Although most bacterial communities were dominated by a few OTUs (Fig. S4b), some communities showed more balanced patterns, as indicated by low Inverse Simpson diversity index (Fig. S4a). Overall, communities were dominated by OTUs affiliated to α-Proteobacteria (78.9% of the reads in average) followed by Bacteroidia (10.4%) and γ-Proteobacteria (10.1%) (Fig. S4c). Sixty per cent of the fruiting bodies showed communities largely dominated by members of the Xanthobacteraceae family (>70% in relative abundance) and in particular by one OTU of the *Bradyrhizobium* genus (Fig. S5). Other communities were characterised by the replacement of *Bradyrhizobium* by another single dominant OTU (e.g. *Chitinophaga*, *Phyllobacterium*, *Pseudomonas*), or the co-dominance of two to eight OTUs (Fig. S5). The massive colonisation of the truffle fruiting body by one OTU or another could not be linked to truffle provenance, as different patterns of colonisation could be retrieved within each site.

However, the structures of the bacterial communities, taking into account all the OTUs detected in truffles fruiting bodies, discriminated depending on fruiting body provenance as indicated by PCoA (Fig. 7a) and PERMANOVA test (Bray–Curtis distance, $R^2 = 0.261$, $P = 0.009$). Maturity again was not a significant effect ($R^2 = 0.027$, $P = 0.3$). Upon analysing the OTUs that were specific to countries (Serbia, Italy, Hungary, Croatia) or collection sites, 24 OTUs (15% of all OTUs detected) and only three genera (*Chryseolina*, *Advenella* and *Solibacillus*) were found in a single region and all except one were found in a single site (Fig. 7b; Table S3). These OTUs were however rare (relative abundance being mostly < 0.02%) and not retrieved in all fruiting bodies collected in the area (Table S3). The exceptions were the OTU 42 belonging to the genus *Chryseolina* (Bacteroidetes) that reached 12.8% of relative abundance in a fruiting body collected in Italy and to *Chitinophaga* OTU 72 found in two fruiting bodies of SER2 (Table S3). In addition, the relative abundance of nine OTUs belonging to eight different genera significantly varied across sites (*F* test, *P*-value adjusted to < 0.05). All nine OTUs were more abundant in fruiting bodies from HUN2 compared with other sites (Fig. 7c). *Pseudomonas* OTU4 massively colonised all fruiting bodies of HUN2; relative abundance varied between 8% and 79% in HUN2, while it was found at an average of 0.9% and rarely exceeded 2% in other fruiting bodies of remaining sites.

Is aroma variability explained by microbes within truffle fruiting bodies?

To determine correlations between VOCs and bacterial composition of individual fruiting bodies, data were analysed with rCCA and loadings projected with covariance values greater than 0.5 were reported. Many of the fruiting bodies did not discriminate, neither did consensus scores discriminate by provenance (Fig. 8a). Five fruiting bodies were clearly separated from others in this analysis: HUN2 (three), ITA1 and SER2 (one each). Note that these five samples were not dominated by *Bradyrhizobium*, instead microbiota had on average a higher richness and had more balanced diversity patterns than other samples ($P < 0.01$, Kruskal–Wallis; Table S4). Several OTUs and five VOCs covaried closely across fruiting bodies (Fig. 8b,c). Relative abundance of these OTUs was up to 4000-fold more abundant in some samples compared with the average in other fruiting bodies. However, their relative abundance remained below 0.1% in all cases. The presence of OTUs of the genera *Luteolibacter*, *Taibaiella*, *Devosia* and *Bosea* were associated with compound RI 814 and was specific for samples HUN2FB (fruiting body) 1 and 3. Similarly, the increased relative abundance of an OTU of the genera *Allorhizobium/Neorhizobium/Pararhizobium/Rhizobium* (ANPR OTU 43) and of the OTU 62 of the *Burkholderiaceae* family was associated with 2-acetyl-5-methylfuran in ITA1FB1. The presence of these OTUs by contrast resulted in a negative covariance with elevated concentrations of methanethiol and DTP. Associations between the presence of specific microorganisms and OAC through this approach were not detected. Despite this, the approach had uncovered non-OAC (or below olfactory detection threshold) that associated with bacterial OTUs.

Discussion

It has not been until recently that differences in the volatile compounds that may indicate geographical differences within *T. magnatum* truffles have been suggested (Strojnik *et al.*, 2020). There is potential for volatile differences by provenance to translate to perceivable differences in truffle aroma, but to date this has not been investigated. In addition, fruiting body maturity and bacterial communities that are suspected of influencing the volatile profile of *T. magnatum* and consequently aroma, have yet to be determined. The current study set out to investigate the underlying VOCs that may result in potential differences in the aroma of *T. magnatum* by means of GC-MS-O, and further confirmed through sensory evaluation. A wide multidisciplinary approach from bacterial communities and fungal spore morphology to volatile chemistry and sensory perception was taken, which had not been attempted until now.

OAC and sensory characteristics of fresh T. magnatum

Some OAC were detected, notably compounds with previously reported highest odour activity values in *T. magnatum*: detection of DTP, 3-methyl butanal, (1H)-pyrroline, 2-methylbutanal, 2,3-butanedione, DMS, DMTS and 1-octen-3-ol corroborated the published literature (Schmidberger & Schieberle, 2017). A few compounds could be additionally suspected as to their identity, inferred by odour character and matching RI: RI 924 (2-acetyl-1-pyrroline) (Schmidberger & Schieberle, 2017) and RI 1181 (2-methylisoborneol) (Mahmoud & Buettner, 2016; Mahmoud & Buettner, 2017). The compound RI 804 was moderate in intensity and, despite lack of identification, it is suspected to be a sulphur compound based on aroma character, but further confirmation is required. Curiously, very few terpenoid compounds were detected by the MS or by olfaction in any of the fruiting bodies, in agreement with Schmidberger & Schieberle (2017) but by contrast with other reports (Gioacchini *et al.*, 2008; Vita *et al.*, 2015, 2018). The causes of such contradiction is currently unknown but may lie in the sampling method of the fruiting bodies. The current study took samples from within the gleba only, to reduce the measurement of VOCs unrelated to truffles. Unfortunately, the literature published on the investigation of white truffles rarely described sample preparation to this extent. Terpenoids nevertheless were not an important aspect of the volatile profile of *T. magnatum*, in particular from an olfactory perspective, and *T. magnatum* might lack key genes for their synthesis (Murat *et al.*, 2018).

At a global level, the drivers of sensory characteristics as measured by RATA were determined through the quantified volatile data using MFA. Sensory characteristics specific to provenance were driven by differences in balance of volatile compound concentrations, not by their presence/absence. The comparisons of the data sets in the current study are unique in that the samples are from different matrices (fruiting bodies vs silicon oil extracts). Despite the differences in physicochemical matrices, the RV coefficient (0.631) showed good agreement between the volatile and sensory data. Oil extracts for sensory assessment was a compromise that gave an acceptable correlation. It is tempting to conclude that the first hypothesis of truffle aroma being dependent on origin at a global level is confirmed. The wide variability seen across individual fruiting bodies within a single site, however, suggests that the apparent discrimination by provenance from the MFA plots should be taken with caution, for reasons explained further below.

Variation of fruiting body volatile profiles are not determined by provenance and maturity

The influence of provenance on truffle VOCs have been suggested previously (Díaz *et al.*, 2003; Gioacchini *et al.*, 2008). The current study attempted to sample truffles from a wide geographical area across southern and eastern Europe. Apart from an apparent similarity in volatile compounds of individual fruiting bodies from Serbia, differences by country of origin were unclear between samples from Italy, Croatia and Hungary. The large average variations seen for ITA1 fruiting bodies compared with those from SER, which had more than a 10-fold less average variation, and may influence sensory perception. Interestingly, despite the two sites from Italy being geographically close, individual fruiting bodies from this country varied the most. This contrasted the published literature, in which fruiting bodies were able to be clustered based on VOCs by provenance both within and across countries (Vita *et al.*, 2018; Strojnik *et al.*, 2020). Volatile markers corresponding to two provenances for *T. magnatum* fruiting bodies have been reported: ethanol, benzaldehyde, 2-methyl-1-butanol and DMS, were characteristic of Slovenian fruiting bodies, whereas anisole, 1,4-dimethoxy-benzene, 1-methoxy-3-methyl-benzene, 1-octen-3-ol, 3-octanone, and 2-methylbutanal were characteristic in Italy (Strojnik *et al.*, 2020). Our results were not as definitive as found in the published literature, in which not even the same compounds detected in the current study were clear contributors that indicated provenance. Compounds such as DMS, benzaldehyde, 1-octen-3-ol, and 2-methylbutanal did not vary to the extent that they were unique to any origin in the fruiting bodies measured in the current study. The disparity in results from that of Strojnik *et al.* (2020), may be influenced by the authors limited number of *T. magnatum* fruiting bodies belonging to one of the geographical locations, and therefore limiting the models. Our results were in line with Vita *et al.* (2018) in which 1-octen-3-ol was not a useful indicator of origin. This contrasted with the black truffle *T. aestivum* for which VOCs were more different across orchards than within (Splivallo *et al.*, 2012). Although our findings led us to conclude that consumers could discriminate the aroma of truffles by provenance, it may only take a single 'rogue' truffle fruiting body with a strong aroma character from within a single site to influence the global aroma of pooled truffle fruiting bodies, creating an 'averaging effect'. While this addresses the practicality of sensory studies, with such high variation of fruiting bod VOCs, a compound could become a marker for a site simply because it is detected at high concentrations in a minority of fruiting bodies. This may have occurred for 2,3-pentanedione in ITA2 and 3-methylbutanal for ITA1, in which concentrations in pooled fruiting bodies were comparatively higher across sites (Table 2), while these two compounds were unusually high in only a few single fruiting bodies from their respective sites.

Maturation of truffles is a complex process, in which changes take place within the fruiting body ascocarps. The changes that take place are not as definitive according to what is measured on the fruiting bodies. Fruiting body mass is independent of maturity for *T. aestivum* (Büntgen *et al.*, 2017), which our results also corroborated. In addition, chemical composition can change with fruiting body maturation, such as increases in monosaccharides and select free amino acids found in *T. melanosporum* (Harki *et al.*, 2006) or reductions in total phenolic content and tannins in *T. aestivum* (Shah *et al.*, 2020). The most common measurement for maturity, albeit unstandardised, is truffle spore melanisation, in which the proportion of spores within the ascocarp that develop pigments and ornamentation increases with maturity (Zeppa *et al.*, 2004). The effect of maturity on VOC profiles has been previously suggested for *T. borchii*, whereby key compounds

that corresponded to specific maturity stages were reported and hypothesised to be derived from fatty acid metabolism as well as isoprenoid biosynthesis (Zeppa *et al.*, 2004). Maturity level in *T. magnatum* however bore no relationship with volatile profiles or bacterial communities and our findings were consistent with the lack of correlation reported in *T. aestivum* fruiting bodies (Splivallo *et al.*, 2012; Molinier *et al.*, 2015). However, due to the current study having only four fruiting bodies that had maturities below 30%, we cannot exclude a link between volatiles and/or bacterial profiles in early stage fruiting bodies and our conclusions on maturity mainly applies to 'intermediate' to 'mature' truffles. In addition, given that the current study did not balance the sampling design to cover a wide range of maturities within each sampling site, it is unknown whether the development of VOCs with maturity can be site dependent. A wider sampling of fruiting bodies to ensure capturing those with low maturities and determine their influence on measurements of VOCs and bacterial community would be warranted.

Bacteria covaried with volatile compounds for a few selected samples

Bacterial community composition of *T. magnatum* was highly variable among fruiting bodies with wide diversity, corroborating with Barbieri *et al.* (2007), no matter their origin. As in other truffle species, members of the *Bradyrhizobium* genus dominated in many fruiting bodies (Antony-Babu *et al.*, 2014; Benucci & Bonito, 2016; Splivallo *et al.*, 2019) but they were replaced by other bacterial taxa in 40% of the fruiting bodies. Similar patterns of variations were found in fruiting bodies of *T. aestivum* (Splivallo *et al.*, 2019) and *T. melanosporum* (A. Deveau *et al.*, unpublished). However, it is noteworthy that non-*Bradyrhizobium* dominant genera differed between *T. aestivum* and *T. magnatum*. OTUs of the genus *Pedobacter*, which were dominant in the bacterial communities of fruiting bodies of certain *T. aestivum*, were found in *T. magnatum* but never dominated. Despite an overall low contribution of provenance on the structure of the bacterial communities (24% of variability), this work revealed the potential existence of bacterial markers of *T. magnatum* origin. Truffles from HUN2 were colonised by a rare *Pseudomonas* OTU from all other sites. Further analysis is required to determine whether it is a peculiarity due to the specific conditions of this site or a more generic phenomenon that can be used to track the origin of truffles on the market. Similar to the VOCs, bacterial communities were not dependent on provenance or fruiting body maturity either, rejecting the second hypothesis.

As many bacteria have the ability to produce truffle VOCs (Vahdatzadeh *et al.* 2016), both of which vary with fruiting bodies, a link between the two have been speculated. To investigate this point, covariance of the microbiota and VOCs were determined. Most covariances were explained by the extreme samples characterised by unusual volatile profiles. It was not surprising that ITA-FB1 was explained by 2-acetyl-5-methylfuran due to its unusually high abundance, making it an anomalous sample, along with four other unidentified VOCs. The close covariance of several OTUs with 2-acetyl-5-methylfuran could suggest that the compound was either derived from bacteria or that some bacteria were favoured by the production of this compound. Several ascomycetes fungi can produce this compound (Ting *et al.*, 2010) but nothing is known about its production by bacteria and its effect on their growth. Although this compound can have a nutty aroma (Burdock, 2010), its level may have been too low to have a role in the aroma of sample ITA1FB1, especially when the compound was not detected through GC-O. Three samples from HUN2 showed high covariance between an unidentified volatile compound (RI_814) with several bacterial OTUs. Further research is needed to identify these specific volatiles and their effect on the development of microbial communities or/and if they are produced by some truffle associated microorganisms.

Our analysis did not show any consistent discrimination of the interaction between bacteria and volatiles by provenance of truffle fruiting bodies. This implied that there was no obvious covariance between *T. magnatum* microbiota and VOCs that were unique to the country of origin, and which led us to reject the third hypothesis. Instead, the variation of truffle fruiting bodies as individuals drove the discrimination of the model and bacteria cannot be used as a predictor of volatile and possible aroma.

The OAC identified in the fruiting bodies through olfactometry did not show any strong correlations with bacterial OTUs measured. It could be concluded that bacteria in *T. magnatum* were not responsible for the synthesis of key OAC, which is by contrast with findings in *T. borchii* (Splivallo *et al.*, 2015). But it is possible that many microbes could produce the same VOCs to the extent that extracting correlations for such information is difficult. This phenomenon has also been demonstrated for thiophene-containing compounds in *T. borchii* (Splivallo *et al.*, 2015). Furthermore, pure cultures of truffle mycelium are also known to produce some of the VOCs that are frequently reported in truffle fruiting, such as 1-octen-3-ol and 3-methyl butanal (Vahdatzadeh & Splivallo, 2018). Indeed, previous studies have attempted to characterise the production of VOCs by specific microbial species *in vitro* (Buzzini *et al.*, 2005; Vahdatzadeh & Splivallo, 2018). The realities of volatile synthesis by microbes within truffle fruiting bodies in nature may be more complex than once thought. With low covariations between OAC and bacteria, other factors may possibly control the synthesis and therefore the variability of VOCs within the fruiting bodies, such as genetic diversity as found in *T. aestivum* fruiting bodies (Molinier *et al.*, 2015). Genetic variability has been reported for *T. magnatum* (Mello *et al.*, 2005) and is further separated into groups based on genetic structure (Belfiori *et al.*, 2020), but its extended influence on volatile profile variation for *T. magnatum* is yet to be determined. Note that the sequencing focused only on bacterial communities and any fungal species that may have been present in the fruiting bodies were not measured. Yeasts have been isolated from *T. magnatum* fruiting bodies (Buzzini *et al.*, 2005) and can induce volatile compounds that are typical of the truffle from L-methionine. Although this alone did not account for the variation in volatile compounds measured in the current study, their presence together with bacteria may be an influencing factor. Specific interactions between yeast and bacterial species can result in volatile compound synthesis (Frey-Klett *et al.*,

2011). Important yeast species that could play a role in such synthesis of key volatile compounds from within the fruiting bodies could possibly have been missed in the current study.

What could be the drivers of volatile/aroma and microbial variation?

Fruiting bodies were measured as 'fresh' as possible within 3 d of harvesting. It is important to note that a reduced time from harvest to measurement does not necessarily indicate absolute fruiting body freshness. Deterioration of fruiting bodies within the soil is possible, which would undoubtedly affect all measurements made. All samples were arbitrarily checked for firmness before fruiting body processing (anecdotal indicator of freshness). Changes taking place within the fruiting body while maintaining firmness is possible. A follow-up study is required to monitor volatile and microbial changes in *T. magnatum* fruiting bodies with storage time.

Truffle availability is season and weather dependent, making harvests unpredictable. Given that *T. magnatum* is notoriously difficult to cultivate in orchards (Riccioni *et al.*, 2016), variability of volatiles and microbiome in the fruiting bodies are dependent on nature. As such, volatile and bacterial community data between the truffle fruiting bodies, even within a single site, largely varied. It is possible that host trees in association with the truffles were a source of the wide variation seen in the current study, as previously suggested for *T. melanosporum* (Culleré *et al.*, 2017). In the current study, the host trees were not provided by truffle hunters. To the best of our knowledge, *T. magnatum* fruiting bodies were all wild, which may have added to variability. Other factors that may play a role in fruiting body variation may be related to soil characteristics, environmental input such as water availability and microclimate weather patterns of the local areas.

During the sample preparation, we opted to remove the peridium of all fruiting bodies before sampling for bacterial community, volatile, and sensory analyses, to focus the investigation on the inner gleba. Bacterial communities drastically differ between gleba and peridium; the microbiome of the peridium tends to be more similar to that of the surrounding soil, making the peridium and gleba two different bacterial habitats (Antony-Babu *et al.*, 2014). As yet, the role of the peridium bacteria on the development of truffle volatiles and therefore aroma is not known and their potential impact cannot be ruled out.

This study was the first to explore underlying relationships between fruiting body maturity, bacterial community, VOCs and sensory perception of *T. magnatum* fruiting bodies. Maturity did not play a role in the variation of VOCs or bacterial communities in the fruiting bodies. The variations in truffle aroma were discernible by chemical means, as well as by OAC and extended to perceptual discrimination of truffle extracts from global aroma assessment by consumers. Key OAC that were ubiquitous across all truffles, drove the differences in aroma perception across sampling sites. Consistent discrimination of fruiting bodies through VOCs and bacterial community by provenance was not found. However, several key bacterial species have suggested a close relationship with key VOCs, albeit nonodour active and the covariances tended to explain only extreme samples. The contribution of bacterial community within fruiting bodies on volatile profiles of *T. magnatum* truffles remains unclear and therefore the underlying drivers of aroma variation of the truffle fruiting bodies requires further research. Our findings are a first step in paving the way for further investigations to determine in detail the relationships between bacterial species, volatile profiles and aroma perception.

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Author contributions

JN and RS contributed to the conception of the experimental concept. JN, RS, and AD contributed to the data collection, analysis, interpretation and preparation of the manuscript. JN, AD and RS contributed equally to this work.

ORCID

Aurélie Deveau https://orcid.org/0000-0001-6266-5241
Jun Niimi https://orcid.org/0000-0002-2642-283X
Richard Splivallo https://orcid.org/0000-0002-7567-6553

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Box and whisker plots of individual fruiting body masses from seven sites.

Fig. S2 Variation of significantly different ($P < 0.05$) odour active compounds measured from individual fruiting bodies across sites.

Fig. S3 Variation of significantly different ($P < 0.05$) odourless/unperceivable compounds measured from individual fruiting bodies across sites.

Fig. S4 Diversity and composition of bacterial communities of *T. magnatum*.

Fig. S5 Composition of bacterial communities of *T. magnatum* at the genus level (relative abundances of the 12 most abundant genera).

Methods S1 Volatile analysis using gas chromatography–mass spectrometry and olfactometry (GC-MS &O).

Methods S2 Microbiome analysis.

Methods S3 Sensory evaluation of truffle extracts using rate all that apply (RATA).

Table S1 List of attributes with definitions derived from GC-O analyses provided to the consumers for RATA.

Table S2 Volatile profile of individual fruiting bodies, measured using the GC-MS.

Table S3 Relative abundance of OTUs in a single geographical area or in a single site.

Table S4 Bacterial richness and Inverse Simpson values of individual truffle fruiting bodies.

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