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SERBIAN BLACK TRUFFLE *TUBER AESTIVUM*: MICROBIOTA AND EFFECTS OF DIFFERENT FREEZING REGIMES ON VOLATILE AROMA COMPOUNDS DURING STORAGE

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Abstract: The use of truffles in food is based mainly on the addition of artificial flavors, aiming to achieve an intense aroma in the products. As truffle is a natural product with nutritional and functional properties, it is important to find an optimal method for truffle storage. As the microbiota contribute to truffle aroma, the bacterial and yeast compositions in the rhizosphere and fruiting body of the truffle and the impact of different freezing methods on the volatile profile of the truffle *Tuber aestivum* during 90 days of the storage were determined. Bacteria and yeasts isolates were identified using 16s rRNA and 18s rRNA. The effect of freezing truffles at -20°C and -80°C with and without previous dipping in liquid N_2 on the volatile compounds was observed using GC/MS. The results demonstrated that the isolated bacteria belonged to the phylum *Proteobacteria*, *Firmicutes* and *Actinobacteria*, and the identified species mainly belonged to *Firmicutes,* genus *Bacillus* sp. Isolated yeasts were identified as *Cryptococcus* sp., *Debaromyces hanseinii*, *Candida fermentati* and *Rhodotorula mucilaginosa*. The GC/MS analysis revealed that frozen truffle samples were richer in the compounds 2-butanone, 2-methyl-butanal, methanethiol and 2-butanol after freezing or during storage. The content of DMS, acetaldehyde, 3-octanone, ethanol, and 2-methyl-1-propanol significantly decreased immediately after freezing. Overall, the gained results indicated that freezing of truffles as a preservation method had profound effects on the volatile compounds, while previous dipping in liquid N_2 showed no significant impact on the volatile profile of truffle *Tuber aestivum*.

Key word: aroma compounds, bacteria, fungi, preservation, yeast.

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Introduction

Truffles are ectomycorrhizal fungi, which belong to the order *Pezizales*, with representatives within *Ascomycota*, *Basidiomycota* and *Zygomycota* (Bonito et al., 2013). Many species and genera are attractive for human consumption, but more attention has been paid to *Tuber* species, especially *Tuber aestivum* Vittad. (Perlińska-Lenart et al., 2020). This truffle species exhibits a much wider distribution than all the others and has been found in almost all European countries, as well as in North Africa (Morocco) and China (Lin et al., 2019; Randazzo et al., 2002; Stobbe et al., 2013; Zambonelli, 2012). Since they develop their fruiting bodies underground (Splivallo et al., 2011), mycorrhizae are established within the roots of *Gymnospermi* and *Angiospermi*, and usually form irregular round fruiting bodies with fleshy consistency or ascocarps (Zambonelli, 2012).

Bacterial communities isolated from truffle ascocarps, their potential participation in nutrition of fruiting bodies, protection from parasitic microorganisms present in the soil, or decomposition of the ascocarps themselves have been the subject of numerous studies. During different stages of development, truffles form symbiotic interactions with bacteria (Archaea and Eubacteria) (Antony-Babu et al., 2014; Barbieri et al., 2007; Gryndler et al., 2013), fungi (yeasts and filamentous fungi) (Buzzini et al., 2005), and viruses (Stielow and Menzel, 2010). Previous studies on the bacterial community of truffles indicate that the surface (peridium) and inner tissues (gleba) are colonized by complex bacterial communities composed of a few hundreds of species, such as *Pseudomonas*, *Bacteroides* and Gram positive bacteria, which dominate in the culturable bacterial communities that can reach up to 10^7–10^8 cells/gram of truffle (Barbieri et al., 2007; Sbrana et al., 2002; Vahdatzadeh et al., 2015). Furthermore, truffle fruiting bodies harbor a diverse microbial community of bacteria, with culture-dependent and culture-independent methods showing that truffles are colonized by *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* (Perlińska-Lenart et al., 2020; Splivallo et al., 2015; Vahdatzadeh et al., 2019). Moreover, yeasts (Buzzini et al., 2005) and filamentous fungi (Pacioni et al., 2007) also represent the part of the truffle microbiota.

The quality of truffles is determined by their specific aromas, which could be different among truffle species and are also responsible for their high economic value all over the world. Today, more than 200 volatile compounds have been isolated and identified from truffle fruiting bodies. Sampling techniques such as direct solvent extraction (DSE), static headspace (SHS), purge and trap (PT), dynamic headspace (DHS), HS-solid phase microextraction (HP-SME) and gas chromatography coupled with mass spectrometry (GC/MS), gas chromatography coupled with flame ionization detector (GC-FID), etc. were used. Most of the truffle volatile compounds were identified as fatty acids, terpenoids, aromatic

compounds, and sulphur-containing compounds (Mustafa et al., 2020). Moreover, studies have shown that many aspects had an influence on the volatile profile of truffles, such as the geographic origin of truffles, different storage conditions, and microbiota (Buzzini et al., 2005; Vahdatzadeh et al., 2019). Recent research on the truffle microbiota indicates that different bacteria are involved in the distinct aroma of truffles through the production of aromatic volatile compounds (Splivallo et al., 2015). Furthermore, correlations between the changes in truffle volatile profiles and specific bacterial classes were obtained during *T. aestivum* storage (Vahdatzadeh et al., 2019). Nonetheless, some studies showed that yeasts isolated from the ascocarps of truffle species can produce volatile organic compounds, indicating that the unique aromas of truffles might also be the result of the yeast community composition (Splivallo et al., 2011).

Freezing is one of the most important methods for retaining food quality during long-term storage. Freezing truffles at a temperature of -20°C and -80°C was among the methods that led to a decrease in enzymatic reactions, preserving the aromatic substances of truffles (Culleré et al., 2013). Saltarelli et al. (2008) found that truffles retained their biochemical and microbiological characteristics during a short storage period at 4°C. A study by Al-Ruqaie (2006) revealed that the freezing process of two truffle species, *Terfezia claveryi* and *Terfezia hakizi*, could be more effective than drying, and concluded that the best preservation method in terms of truffle quality was blanching in a 4% NaCl solution and storing at -18°C. Furthermore, Jaworska and Bernás (2009) proposed a maximum storage period of 4 months for frozen, unblanched mushrooms. Moreover, Culleré et al. (2013) determined differences in the volatile profiles of fresh and frozen truffles.

The study of the bacterial and yeast microbiota and volatile profile of truffles, as well as an appropriate preservation method that preserves aromatic compounds during truffle storage, has been a great challenge in recent years. Therefore, the aim of this study was to determine the bacterial and yeast composition in the rhizosphere and fruiting body of the Serbian truffle, as well as the impact of freezing at different low temperatures with and without liquid N_2 on the volatile profile of the truffle *T. aestivum* by means of HS combined with GC-MS/FID during 90 days of the storage.

Material and Methods

Sample collection

Truffles were collected manually, with the help of a trained dog in northern Serbia, in the area of Srem, in broad-leaved Bojčin forest (44°73'N and 20°15'E), where linden (*Tilia tomentosa*) and oak (*Quercus robur*) predominate. The analyzed samples were black summer truffles (*T. aestivum*), collected randomly

(10 samples) in early August of 2018. The post-harvested truffles were wrapped in a highly absorbent blotting paper and transported in refrigerated conditions in insulated boxes with ice packs to the laboratory, where they were analyzed upon arrival, while the rest of the samples were frozen in appropriate conditions.

Isolation of different microorganisms from truffles

Fresh and undamaged ascocarps of *T. aestivum* (i.e., no signs of insect larval galleries, dry damage, or injuries from animals or harvesting equipment) were carefully washed with sterile water, the surface of the ascocarps was removed under sterile conditions, and the gleba and ascocarp surface were submerged in sterile saline (0.86% NaCl). For bacterial isolation, the samples of gleba and ascocarp surface were homogenized with vortex and appropriate dilutions were plated on Tryptone Soya Agar (TSA, Merck, Germany) and cetrimid agar for isolation of bacteria and *Pseudomonas*, respectively. Plates were incubated at 30°C for 48 h (Perlińska-Lenart et al., 2020). Single colonies were sub-cultured on appropriate agar to obtain pure cultures, which were stored at -80°C in medium with 20% of glycerol.

From the same material, YEPG agar was used to isolate yeasts (Zacchi et al., 2003). The plates were incubated at 25°C for 48 h. Then, distinct single colonies were picked out and sub-cultured a few times onto fresh YME agar plates to obtain pure cultures. The isolated yeasts were stored at -80°C in a suitable medium with 20% of glycerol.

DNA isolation and identification of microorganisms

Total DNA of bacterial isolates was isolated by using a commercial kit for DNA isolation (Quick-DNA Fungal/Bacterial Kit, Zymo Research) according to the manufacturer's instructions. For DNA isolation of yeast isolates, a commercial kit (DNA Isolation Kit for Cells and Tissues, Merck, Darmstadt*)* was used, according to the manufacturer's instructions. Identification of selected bacterial isolates was performed by sequencing the 16s rRNA gene using specific primers P1 16S and P2 16S, whereas identification of yeast isolates was performed using specific primers for 18s rRNA (NS1 and NS2), which are listed in Table 1. Taq DNA polymerase (Kapa Biosystems Inc., Boston, USA) was used to amplify the 16s rRNA and 18s rRNA genes using a PCR system thermal cycler (GeneAmp PCR System 2700, Applied Biosystems, Foster City, CA, USA). A 1% agarose gel at a constant voltage of 1–10 V/cm was used to verify the PCR products. PCR products were purified by using a Thermo Scientific PCR Purification Kit (Thermo Scientific, Lithuania) according to the manufacturer's instructions. The purified PCR products were sequenced by the Macrogen Sequencing Service (Macrogen

Europe, Amsterdam, Netherlands). The BLAST algorithm was used for analyzing of nucleotide sequences (Altschul et al., 1997) http://www.ncbi.nlm.nih.gov/BLAST.

Table 1. Sequences of primers.

Name	Nucleotide sequence	Reference
P1 16S	5' -GAGAGTTTGATCCTGGC-3'	Jovčić et al., 2009
P2 16S	5' -AGGAGGTGATCCAGCCG-3'	Jovčić et al., 2009
NS1	5'- GTAGTCATATGCTTGTCTC-3'	White et al., 1990
NS2	5'- GGCTGCTGGCACCAGACTTGC-3'	White et al., 1990

Freezing of truffle samples

Volatile compounds were detected in fresh samples, frozen at -20°C (Deep Freezer Samsung, Germany) and -80°C (Thermoscientic, HERA freeze, Germany), and in samples immersed in liquid nitrogen and stored at -20°C and -80°C. Aromatic compounds were determined immediately after freezing and after 30, 60, and 90 days of storage.

Truffle preparation for GC/MS

The fresh samples were ground in a laboratory mill and transferred to 20-ml HS vials. Frozen samples at -20 and -80°C were transferred to a cold room and analyzed. Samples were heated at 100°C for 20 min using the following program: incubation temperature: 100°C; incubation time: 1200 s; syringe temperature: 110°C. For the analysis of volatiles, 2000 μL of the generated vapor was extracted from the vial and injected directly into the gas chromatograph using a heated gas-tight syringe (Nikolić et al., 2018, 2019).

Gas chromatography-flame ionization detector (GC-FID) and gas chromatography-mass spectrometry (GC/MS) analyses

Gas chromatography (GC) and gas chromatography/mass spectrometry (GC-MS) analyses were performed using an Agilent 7890A GC equipped with an inert 5975C XL EI/CI mass spectrometer detector (MSD) and a flame ionization detector (FID) connected to the makeup through a 2-way capillary flow technology splitter. HP-Innowax fused silica capillary column (30 m × 0.32 mm × 0.25 μm). For the HS analyses, 2000 μL of the generated vapor was extracted from the vial and injected directly into the gas chromatograph using a heated gas-tight syringe (110°C). The column temperature program began at 35°C (5 min), then increased to 65°C at 3°C min^{-1}, and finally reached 225°C at a rate of 10°C min^{-1} and lasted for 4 min. Helium

was used as the carrier gas at 3.0 mL/min (constant flow mode). The sample was analyzed in the split less mode with a split ratio of 3:1. The injector temperature was 250 °C and the detector temperature was 300 °C. MS data were acquired in EI mode with a scan range of 40–550 *m/z*, a source temperature of 230 °C, and a quadrupole temperature of 150 °C, and the solvent delay was 3 minutes.

The components were identified based on their retention index and comparison with reference spectra (Wiley and NIST databases) as well as by the retention time locking (RTL) method and the RTL Adams database. The retention indices were determined experimentally using the standard method of Van Den Dool and Kratz (1963), involving retention times of *n*-alkanes injected after the sample under the same chromatographic conditions. The relative abundance of the *n*-alkanes was calculated from the signal intensities of the homologues in the GC-FID traces.

Results and Discussion

Identification of bacteria and yeasts

The results of identification of isolates from truffles, based on 16s rRNA sequencing, are presented in Table 2.

Table 2. Identified bacteria from truffles.

Isolates from ascocarps	% of abundance	Isolates from gleba	% of abundance
Bacillus sp.	56.3	*Arthrobacter* sp.	3.1
Microbacterium sp.	3.1	*Pseudomonas* sp.	9.3
Pseudomonas sp.	3.1	*Bacillus* sp.	6.2
Staphylococcus sp.	9.3	*Enterococcus* sp.	3.1
		Brevibacterium sp.	6.2

Totally 32 bacteria were isolated from truffle, out of which 23 isolates from ascocarps and 9 from gleba. The predominant genus of the selected isolates was *Bacillus* spp. (20 isolates), out of which 18 isolates were isolated from the ascocarp and 2 from the gleba. Other selected isolates were classified into 6 genera: *Pseudomonas* sp. (5 isolates), *Staphylococcus* sp. (2 isolates), *Brevibacterium* sp. (2 isolates), *Microbacterium* sp. (1 isolate), *Arthrobacter* sp. (1 isolate), and *Enterococcus* sp. (1 isolate).

In a previous study (Barbieri et al., 2005), the bacterial isolates of *T. borchii* were affiliated to the γ-*Proteobacteria* class, whereas some isolates belonged to the Bacteroidetes group and Gram positive bacteria, mostly *Bacilliaceae*. Furthermore,

sequence analysis of the bacterial isolates of *T. magnatum* identified *Proteobacteria* comprising α, β and γ subdivision, the *Bacteroidetes* group and the *Acinetobacter* and *Firmicutes* phyla (Barbieri et al., 2007). The difference in the obtained results could be explained by the study of Splivallo et al. (2015), where changes in the microbiota of truffles were noticed during storage. The bacteria isolated on the first day of storage mostly belong to the class of α-*Proteobacteria* and *β-Proteobacteria,* while after 6 days of storage, an increase in abundance of the *Firmicutes* was observed. The genus *Bacillus* belongs to the phylum *Firmicutes*, which was the dominant bacterial microbiota in our results. Moreover, differences were observed between truffle species in some bacterial microbiota, such as *Betaproteobacteria*, *Gammaproteobacteria*, and *Bacteroidetes,* which were more abundant in *T. borchii* than in *T. melanosporum* and *T. magnatum* (Vahdatzadeh et al., 2015). Moreover, a specific comparison of the gleba and peridium compartments during maturation showed that some phyla, such as *Firmicutes*, increased significantly in the gleba of fully mature ascocarps, while remaining stable in the peridium (Antony-Babu et al., 2014).

The present bacterial population could be related to the production of volatile compounds released by the interaction of truffles and bacteria. The absence or rare occurrence of microorganisms that form a specific aroma indicates that the aromatic compounds could be synthesized by the truffle itself or during its sexual phase (Splivallo et al., 2015).

Yeasts were isolated from the surface of the ascocarp and from the gleba and 4 out of 7 isolates were selected after microscopic examination. The yeast isolates were identified as *Cryptococcus* sp., *Debaromyces hanseinii*, *Candida fermentati* and *Rhodotorula mucilaginosa* based on 18s rRNA. Previous studies have revealed that the yeast microbiota consist of five species: *Cryptococcus albidus*, *Cryptococcus humicola*, *Rhodotorula mucilaginosa*, *Debaryomyces hansenii*, and *Saccharomyces paradoxus* (Zacchi et al., 2003). The yeasts *Candida saitoana*, *Deb. hansenii*, *Cryptococcus*, *Rhodotorula* and *Trichinosporun* were also isolated from *T. melanosporum* and *T. magnatum* (Buzzini et al., 2005). Interestingly, *Cryptococcus* sp., *R. mucilaginosa*, *D. hansenii*, and *Saccharomyces* sp. were also isolated from *T. melanosporum*, *T. magnatum*, or *T. aestivum* and might therefore be common to distinct truffle species (Vahdatzadeh et al., 2015). The obtained results indicate that the yeast microbiota might vary between different truffle species, as well as within the part of the tissue.

Volatile compounds in fresh and frozen samples of *T. aestivum*

The volatile organic compounds of fresh and frozen truffles during storage are shown in Tables 3, 4, 5, 6 and 7. The results of the volatile organic compounds presented represent the percentage of each compound in relation to the total content of volatile compounds detected. A total of 57 volatile compounds were detected, of

which 19 were selected as significant volatile compounds. The selected compounds detected in the truffle samples were sulphur-containing (3), aldehydes (4), ketones (4), alcohols (6) and furans (2).

The presence of sulphur compounds was determined in all truffle samples, regardless of the regime of freezing, with methanethiol and dimethyl sulphide (DMS) accounting for a significant share in relation to other sulphur compounds. Previous studies have shown that these compounds are essential components contributing to the aroma of black truffles in Europe and Asia (Chen et al., 2019; Culleré et al., 2010). The content of DMS decreased significantly on day 0 of freezing. Thereafter, the content was slightly reduced and maintained at a similar level in all freezing regimes until day 90 of storage. After freezing, the content of methanethiol decreased in all freezing regimes and a slight increase was observed after 90 days of storage, especially when freezing at -20°C. After 90 days of storage, the level of methanethiol was more than three times higher than on day 0, which could be explained by the decrease of other compounds in the overall aromatic composition.

Table 3. Volatile sulphur compounds in fresh and frozen truffles at appropriate temperatures.

No.	Volatile compounds	RI	Fresh truffle	Days	Relative share during storage (%)			
					-20°C	Liquid N_2 -20°C	-80°C	Liquid N_2 -80°C
1.	Methanethiol	666	0.4	0	0.2	0.2	0.2	0.2
				30	0.8	1.4	1.1	1.1
				60	0.5	0.8	1.0	0.9
				90	1.2	0.8	0.9	0.7
2.	Dimethyl sulphide	737	3.97	0	0.3	0.3	0.3	0.3
				30	0.2	0.2	0.1	0.3
				60	0.2	0.2	0.1	0.3
				90	0.2	0.1	0.1	0.3
3.	3-(Methylthio) propanal	1724	0.17	0	0.05	0.03	0.02	0.01
				30	/	/	/	/
				60	/	/	/	/
				90	/	/	/	/

Sulphur-containing compounds derive from the catabolism of L-methionine, their major precursor (Spinnler et al., 2001). Sulphur compounds constituted the largest group of volatile compounds (thiols, thioesters, sulphides, thioalcohols and thiophenones), but generally they have a very low olfactory detection limit,

although they represent major contributors to the final aroma of truffle fruiting bodies. Methanethiol, also known as methyl mercaptan, is a product of methionine degradation. It has an unpleasant odor and a low threshold value of approximately 1 ppb (Devos et al., 1990). Volatile compounds such as DMS were present in most truffle species and were probably formed as a result of the Ehrlich pathway. These sulphur compounds could be conditioned by microbial activity (Martin et al., 2010; Splivallo et al., 2011), whereas DMS could be formed as a result of yeast activity (Buzzini et al., 2005). Furthermore, sulphur compounds, 2-methylbutanal, 3-methylbutanal, 2- methylbutan-1-ol, 3-methylbutanol and oct-1-en-3-ol were detected in most truffle species (Vahdatzadeh et al., 2019).

Table 4. Volatile aldehydes in fresh and frozen truffles at appropriate temperatures.

No.	Volatile compounds	RI	Fresh truffle	Days	Relative share during storage (%)			
					-20°C	Liquid N_2 -20°C	-80°C	Liquid N_2 -80°C
1.	Acetaldehyde	686	6.32	0	3.0	3.0	3.0	3.0
				30	0.8	2.1	1.2	1.2
				60	1.7	1.8	2.8	2.7
				90	1.1	1.2	1.3	1.1
2.	2-methyl-1- propanal	816	5.26	0	11.1	11.1	11.1	11.1
				30	7.9	19.1	8.7	10.6
				60	7.6	7.2	13.4	12.8
				90	0.5	0.4	0.4	0.3
3.	2-methyl-butanal	889	9.55	0	10.6	10.6	10.6	10.6
				30	13.8	15.0	17.4	13.8
				60	14.7	13.2	15.8	14.7
				90	13.1	7.6	11.7	13.1
4.	3-methyl-butanal	911	7.43	0	7.6	7.6	7.6	7.6
				30	0.4	0.2	0.3	0.4
				60	0.4	0.8	0.3	0.4
				90	9.8	6.6	9.3	9.8

The main volatile aldehydes were detected in all truffle samples: acetaldehyde, 2-methyl-propanal, 2-methyl-butanal and 3-methyl-butanal. The concentration of acetaldehyde decreased two times immediately after freezing compared to fresh truffle, and it continued to decrease under all storage conditions. The content of 2-methyl-propanal doubled after freezing the truffle samples, and after 90 days of storage the relative concentration dropped approximately twenty times. Likewise, the concentration of 2-methyl-butanal in frozen truffles was higher on day 0 and increased slightly during storage, except for truffle samples treated with liquid nitrogen and stored at -20°C on day 90 of storage where concentration decreased. For the component 3-methyl-butanal, a slight increase was observed after 90 days of storage in all freezing regimes.

Aldehyde compounds such as 2-methylbutanal and 3-methylbutanal represent common constituents of all truffle aromas (Costa et al., 2015) and have been reported as predominant compounds in the volatile profile of truffles, with qualitative fluctuations depending upon variables such as truffle type and geographical origin (Fiecchi et al., 1967; Gioacchini et al., 2005).

Table 5. Volatile ketones in fresh and frozen truffles at appropriate temperatures.

No.	Volatile compounds	RI	Fresh truffle	Days	Relative share during storage (%)			
					-20°C	Liquid N_2 -20°C	-80°C	Liquid N_2 -80°C
1.	2-Butanone	880	1.67	0	48.7	48.7	48.7	48.7
				30	35.4	29.3	32.4	39.4
				60	39.8	43.3	24.0	39.8
				90	39.2	41.9	37.5	35.2
2.	2,3-Butanenedion	994	0.56	0	0.2	0.2	0.2	0.2
				30	0.1	0.2	0.1	0.1
				60	0.1	0.1	0.2	0.1
				90	0.1	0.1	0.1	0.1
3.	2,3-Pentanedion	1056	0.67	0	0.4	0.4	0.4	0.4
				30	0.1	0.2	0.2	0.1
				60	0.2	0.1	0.3	0.2
				90	0.2	0.1	0.1	0.2
4.	3-Octanon	1257	2.49	0	0.1	0.1	0.1	0.1
				30	0.2	0.2	0.3	0.3
				60	0.2	0.9	0.3	0.2
				90	0.6	0.8	0.7	0.7

Ketones are very important components of the aromatic profile of truffles, synthesized via the fatty acid *β*-oxidation pathway. The results show that the dominant ketones appeared to be: 2-butanone, as the predominant volatile ketone component, 2,3-butanedione, 2,3-pentanedione and 3-octanone. The concentration of 2-butanone increased multiple (24) times in all frozen truffle samples after freezing on day 0 and remained at the same level during 90 days, while the concentration of 2,3-butandione reduced twice on day 0 and was maintained during 90 days of storage. The compounds 2,3-pentanedione and 3-octanone slightly decreased after freezing and remained at the same level during the storage period. The component 2-butanone was detected in different *Tuber* species and was found in the fruiting bodies of *T. magnatum*. The results of the study by Strojnik et al. (2020) indicate that component 2-butanone is a quality marker, since samples with low amounts (<15%) had an unpleasant rotten odor, while samples with high amounts of 1-octen-3-ol (≤80%) had a pleasant odor. Likewise, similar ketone compounds were detected in the fresh truffle species *T. sinensi*, *T. sinoalbidum* and T. *sinoexcavatum* (Feng et al., 2019).

Table 6. Volatile alcohols in fresh and frozen truffles at appropriate temperatures.

No.	Volatile compounds	RI	Fresh truffle	Days	Relative share during storage (%)			
					-20°C	Liquid N_2 -20°C	-80°C	Liquid N_2 -80°C
1.	Ethanol	922	18.36	0	0.7	0.7	0.7	0.7
				30	1.2	1.3	1.5	1.2
				60	1.1	2.1	0.9	1.1
				90	0.8	2.6	1.4	0.8
2.	2-butanol	1023	17.90	0	14.7	14.7	14.7	14.7
				30	29.3	21.9	28.6	29.3
				60	29.2	24.1	34.0	29.2
				90	24.1	22.0	19.4	22.1
3.	1-propanol	1034	0.64	0	0.5	0.5	0.5	0.5
				30	1.1	1.6	0.7	1.1
				60	1.1	0.4	1.2	1.1
				90	0.4	0.5	0.4	0.4
4.	2-methyl-1-propanol	1089	1.02	0	0.5	0.5	0.5	0.5
				30	0.3	0.3	0.6	0.3
				60	0.4	0.6	0.5	0.4
				90	0.5	0.6	0.7	0.4
5.	2-methyl-1-butanol	1215	12.13	0	0.6	0.6	0.6	0.6
				30	1.1	0.2	2.2	0.3
				60	1.0	0.8	2.1	0.3
				90	1.3	0.8	2.0	0.8
6.	1-octen-3-ol	1467	2.49	0	2.1	2.1	2.1	2.1
				30	2.3	4.9	2.8	5.3
				60	2.4	2.4	2.7	4.6
				90	2.6	2.5	2.4	3.6

In terms of alcohol content, 6 volatile compounds were monitored during 90 days of storage: ethanol, 2-butanol, 1-propanol, 2-methyl-1-propanol, 2-methyl-1-butanol, 1-octen-3-ol. The obtained results showed that 2-butanol was the predominant volatile alcohol compound. The freezing regimes had no effect on the content of 2-butanol on day 0. The highest concentration of 2-butanol was obtained after 90 days of storage at -20°C, while the lowest concentration was noticed at -80°C. Nevertheless, the concentration of 2-butanol was higher by 50–70% in all freezing regimes, compared to fresh truffle. Similar results were gained for 1-octen-3-ol, which is believed to have a signalling effect on plants (Barbieri et al., 2005). The content of 1-octen-3-ol was reduced in all freezing regimes on day 0 comparing to fresh truffle (from 2.49% to 2.1%). After 30 days of storage, the

content of this volatile component had doubled under freezing conditions with liquid N_2 (-20°C and -80°C). The content of 1-octen-3-ol in frozen truffle with N_2 at -80°C was higher than in fresh truffle, while the content of this solution was similar to fresh truffle in all other regimes. Based on the obtained results, it is believed that freezing in liquid N_2 has a positive effect on 1-octen-3-ol, by increasing the content of this solution comparing to fresh truffle. This alcohol compound is generally the flavor of fresh mushrooms found in human breath and sweat (Rajarathnam and Shashirekha, 2003).

The concentration of 2-methyl-1-butanol decreased 20 times comparing to fresh truffle on day 0 and then it showed a growing trend for samples stored at -20°C and -80°C during 90 days of storage. The concentration of this component was higher in freezing regimes without liquid N_2 after 60 and 90 days of storage. The gained results indicate that freezing with liquid N_2 had a negative impact on the concentration of 2-methyl-1-butanol. This alcohol has been reported to be the major contributor to the final aroma of *T. melanosporum*. Furthermore, 2-methyl-1-butanol is derived from fatty acid catabolism and is well represented among truffle species. Moreover, alcohols can be oxidized to the corresponding component; 2-butanol could be transformed into 2-butanone and further transformation may lead to 2-methylpropanal, 3-methylpropanal or 2-butanal (March et al., 2006).

Table 7. Volatile furans in fresh and frozen truffles at appropriate temperatures.

No.	Volatile compounds	RI	Fresh truffle	Days	Relative share during storage (%)			
					-20°C	Liquid N_2 -20°C	-80°C	Liquid N_2 -80°C
1.	Furan	804	0.97	0	0.4	0.4	0.4	0.4
				30	0.4	0.8	0.5	0.5
				60	0.4	0.4	0.7	0.6
				90	0.1	0.1	0.1	0.1
2.	2-Furanmethanol	1673	0.2	0	0.2	0.2	0.2	0.2
				30	0.2	0.3	0.5	0.2
				60	0.2	0.6	0.2	0.1
				90	0.4	0.8	1.0	0.4

Studies have shown that eight carbon compounds contribute to the specific truffle aroma, since they impart a characteristic odor to the mushroom (Combet et al., 2006). Also, authors Splivallo et al. (2011) have detected alcohol compounds in several truffle species (*T. borchii* and *T. indicum*). Applied freezing regimes after 90 days contributed to the formation of truffle aroma, since the content of these alcohols increased during storage compared to the content of fresh truffles.

The obtained results show that the furan derivatives were furan and 2-furanmethanol. Of these two compounds, furan showed the greatest differences in concentration depending on the working conditions. After freezing on day 0, the concentration of furan decreased by 50% proportionately in all freezing regimes in regard to fresh truffle. The concentration of this compound after 90 days of storage was nine times lower than in fresh truffle.

The concentrations of 2-furanmethanol were unchanged in all freezing regimes on day 0. However, after 60 and 90 days of storage, the concentration of this compound in the regime with liquid N_2 at -20°C was three times higher than in fresh truffle. Furthermore, after 90 days of storage at -80°C, the concentration of 2-furanmethanol was five times higher than that of fresh truffle, while the concentration was two times higher when stored with liquid N_2 at -80°C, indicating that the freezing regime without liquid N_2 had a better effect on the concentration of this compound. Considering furans and furanones, Díaz et al. (2003) detected 2-pentylfuran in *T. escavatum*, *T. aestivum* and *T. melanosporum* via SPME. These compounds have a characteristic odor of fruits, green and earthy plants with a nuance similar to vegetables. Effenberger et al. (2019) found that 2 (5H) furanone had typical odor of caramel or burned sugar at low concentrations. Furaneol, often described as an attribute of "caramel" and "sweet" scent (Culleré et al., 2010; Ong and Acree, 1998), is associated with sweetness, along with γ-decalactone, which other authors have described as having "fruity" and "sweet" notes (de Andrade et al., 2017).

In this study, among 19 selected significant volatile compounds, 2-butanone and 2-butanol were quantitatively dominant and accounted for more than 50% of the total aroma in all frozen truffle samples.

Conclusion

Most of the identified bacteria belonged to the genus *Bacillus* sp., 56.3% from the ascocarp and 6.2% from the gleba. Other isolates belonged to *Pseudomonas* sp., *Staphylococcus* sp., *Brevibacterium* sp., *Microbacterium* sp., *Arthrobacter* sp., and *Enterococcus* sp. Yeast isolates were identified as *Cryptococcus* sp., *Debaromyces hanseinii*, *Candida fermentati* and *Rhodotorula mucilaginosa*. Further analysis of different samples from other regions in Serbia is required to better understand the microbiota of *T. aestivum.*

The GC/MS analysis revealed differences in the volatile profile of fresh and frozen truffles, both immediately after freezing and during 90 days of storage. The frozen samples were richer in the compounds 2-butanone, 2-methyl-butanal, methanethiol and 2-butanol after freezing or during storage, of which 2-butanone and 2-butanol quantitatively dominated and accounted for more than 50% of the total aroma in all frozen truffle samples. The content of DMS, acetaldehyde, 3-

octanone, ethanol, and 2-methyl-1-propanol significantly decreased immediately after freezing. Freezing could be a good preservation method regardless of the temperature of the freezing regime. Likewise, liquid N_2 generally showed no significant effect on the volatile profile of truffles.

Further analysis could provide better insight into the microbiota of truffles from other regions of Serbia, as well as the influence of bacterial and yeast microbiota on the volatile profile of truffles. Furthermore, the application of other techniques for the determination of volatile compounds, their quantification, as well as sensory analysis of fresh and frozen truffle samples, would provide significantly better insight into the changes in the relevant volatile compounds and the volatile profile of truffles.

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SRPSKI CRNI TARTUF *TUBER AESTIVUM*: MIKROBIOTA I UTICAJ RAZLIČITIH REŽIMA SMRZAVANJA NA ISPARLJIIVA AROMATIČNA JEDINJENJA TOKOM SKLADIŠTENJA

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R e z i m e

Primena tartufa u hrani se uglavnom zasniva na dodavanju veštačkih aroma, sa ciljem postizanja intenzivne arome tartufa u proizvodima. S obzirom na to da su tartufi proizvodi sa nutritivnim i funkcionalnim karakteristikama, važno je pronaći optimalan način za skladištenje tartufa. Pošto mikrobiota doprinosi aromi tartufa, sastav bakterija i kvasaca u rizosferi i plodonosnom telu srpskog tartufa, kao i uticaj različitih metoda smrzavanja na isparljiva jedinjenja tartufa *Tuber aestivum* ispitivan je tokom 90 dana skladištenja. Bakterije i kvasci su izolovani iz svežeg tartufa i izolati su identifikovani korišćenjem 16s rRNK i 18s rRNK. Uticaj smrzavanja tartufa na -20°C i -80°C sa i bez prethodnog potapanja u tečni N_2 na sadržaj isparljivih jedinjenja ispitivan je korišćenjem GC/MS. Rezultati ispitivanja su pokazali da izolovane bakterije pripadaju carstvu *Proteobacteria*, *Firmicutes* i *Actinobacteria*, pri čemu identifikovane vrste uglavnom pripadaju *Firmicutes,* rod *Bacillus* sp. Izolovani kvasci su identifikovani kao *Cryptococcus* sp., *Debaromyces hanseinii*, *Candida fermentati* i *Rhodotorula mucilaginosa*. Analiza GC/MS je ukazala na razlike u profilu isparljivih jedinjenja svežeg i smrznutog tartufa. Smrznuti uzorci su više sadržali komponente kao što su 2-butanon, 2-metil-butanal, metanetiol i 2-butanol nakon smrzavanja ili tokom skladištenja. Sadržaj DMS, acetaldehida, 3-octanona, etanola, 2-metil-1-propanola značajno se smanjio odmah nakon smrzavanja. Dobijeni rezultati ukazuju da smrzavanje tartufa kao metoda konzervacije ima značajan uticaj na isparljiva jedinjenja, pri čemu potapanje u tečni N_2 nije pokazalo značajan uticaj na isparljiva jedinjenja tartufa *Tuber aestivum*.

Ključne reči: aromatična jedinjenja, bakterije, gljive, konzervacija, kvasci.

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