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Assessment of Microbiological Properties, Mycotoxins, and Heavy Metals in Underprized Raw Kalahari Truffles Sold in Namibia

T.A. Hainghumbi [1], W. Embashu [2], K.K.M. Nantanga [1*], N.P. Kadhila [2], L. Iipumbu [3]

1. Department of Food Science and Systems, Faculty of Agriculture, Engineering and Natural Sciences, University of Namibia
2. Multidisciplinary Research Services, University of Namibia
3. Toxicology and Residue Analysis Section, Central Veterinary Laboratory, Directorate of Veterinary Services-Ministry of Agriculture, Water and Land Reform

HIGHLIGHTS

- Total aerobic count of unwashed truffles ranged from 4.4 to 7.3 log Colony Forming Unit (CFU)/g.
- Ochratoxin A levels in unwashed truffles ranged from 0.1 to 48.5 µg/kg.
- Total aflatoxin levels were 26.3 to 27.5 µg/kg, while zearalenone levels ranged from 45.0 to 9,680 µg/kg.

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Acronyms and abbreviations
CFU=Colony Forming Unit

ABSTRACT

Background: Kalahari truffle (*Kalaharituber pfeilii*) is found in the Kalahari desert and nearby regions (Africa). This study assessed the microbiological quality and safety, mycotoxins, and heavy metals contents of raw Kalahari truffle sold in Namibia.

Methods: Batches of Kalahari truffles were purchased from informal markets and different vendors in Namibia. Total aerobic, coliform, yeast, and moulds counts, and *Salmonella* were assessed. Also, some mycotoxins and heavy metals were determined. Data were analyzed using SPSS Statistics Software, Version 25.

Results: Total aerobic count of unwashed truffles ranged from 4.4 to 7.3 log Colony Forming Unit (CFU)/g. Total coliform counts detected in truffles were 6.0 log CFU/g. *Salmonella* was not detected. Doxynivalenol was the most prevalent mycotoxin. Fumonisin B_1 levels ranged from 17.4 to 142.1 µg/kg. Ochratoxin A levels in unwashed truffles ranged from 0.1 to 48.5 µg/kg. Total aflatoxin levels were 26.3 to 27.5 µg/kg, while zearalenone levels ranged from 45.0 to 9,680 µg/kg. The iron content was up to 746.72 mg/kg. Cadmium and zinc were detected in the studied samples, but mercury and nickel were no detectable in any samples.

Conclusion: The studied truffle samples were safe in terms of *Salmonella*, mercury, and nickel. However, some of the detected microorganisms, mycotoxins, and heavy metals in underprized Kalahari truffles may impair the safety, shelf life, and human health. Thus, they should be subjected to appropriate processing before consumption.

Introduction

Truffles are edible ectomycorrhizal, hypogenous mushrooms found in many countries such as Italy, Germany, France, USA, China, Saudi Arabia, Namibia, Botswana, and Zambia. In some developing countries such as those in southern Africa, truffles are seasonal and are harvested for primarily household consumption and for sale at in

* Corresponding author (K.K.M. Nantanga)
E-mail: knantanga@unam.na
ORCID ID: https://orcid.org/0000-0002-1776-3595

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formal markets and along the main roads. Kalahari truffle (*Kalaharituber pfeilii*) is found in the Kalahari desert and nearby regions (Africa). They are underpriced as compared to other truffles in other parts of the world (Álvarez-Lafuente et al., 2018; Trappe et al., 2008).

Fresh mushrooms including truffles have a high moisture content and water activity which make them an ideal medium for microbial growth. For instance, Venturini et al. (2011) detected total microbial counts in the range from 4.4 to 9.4 log Colony Forming Unit (CFU)/g and total coliform bacteria in 23.4% of different mushroom species sampled. Ezekiel et al. (2013) detected toxigenic moulds in dried mushrooms. Consequently, the presence of bacterial and unwanted fungal populations in fresh mushrooms can cause quality deterioration and reduce the shelf-life of fresh mushrooms (Venturini et al., 2011).

Moreover, proliferation of toxigenic moulds in foodstuff could lead to the production of mycotoxins. Mycotoxins have a negative impact on the immune system, liver, kidneys, and blood whereas some mycotoxins are found to be carcinogens (Ünüsan, 2019). Another safety aspect of mushrooms is the presence of heavy metals. High levels of heavy metals can cause health complications in humans. These include neurotoxicity, binding to proteins and enzymes and the promotion of oxidative stress that damageimportant molecules such as the DNA, proteins, and lipids (Paithankar et al., 2021). Heavy metals such as lead (Pb), cadmium (Cd), manganese (Mn), copper (Cu), nickel (Ni), and cobalt (Co) have been detected in wild fresh and unprocessed mushroom species (Sarikurkcu et al., 2011). To preserve and understand the safety of these seasonal and telluric delicacies, this study investigated the microbial quality, mycotoxins, and heavy metals in wild growing edible raw Kalahari truffle, one of the least studied truffles.

Materials and methods

Sample collection and treatment

From May to June 2018, eight batches of fresh raw Kalahari Desert truffles (*Kalaharituber pfeilii*) were purchased from different vendors at the main informal markets where Kalahari truffles are seasonally sold in Namibia (Figure 1), including Omuthiya, Ondangwa, and Casablanca open markets. One batch was obtained from each of a total of eight vendors. Six batches (T_1, T_2, T_3, T_4, T_7, and T_8) of truffles were purchased during the peak of the growing season (mid May 2018) while batches T_5 and T_6 were purchased near the end of growing season (around June). Truffle batches (T_1-T_6) were bought from different vendors at Omuthiya gwIipundi in Oshikoto region whereas batch T_7 was bought from Ondangwa in Oshana region. Batch T_8 was purchased from Casablanca, Oshikoto Region.

Each batch was divided into two portions. One portion from each batch was washed under running water whereas the other remained unwashed. Each batch therefore resulted into two samples (washed and unwashed). Both unwashed and washed truffles were then sliced and spread on separate trays. Drying was done at ambient conditions in the Food Processing Laboratory for five consecutive days. Dried truffles were then ground and kept frozen. A portion of one sample (T_6; fresh) was frozen without drying.

Moisture content

Moisture content of truffles was determined using the AACC International Method 44-15.02 (AACC International, 1999). Approximately 2 g of sample was weighed and heated in an oven at 130 °C for 60 min and cooled for 45 min in a desiccator before weighing and moisture determination.

Microbial analyses

-Aerobic plate count

Total aerobic plate count was carried out following a method described by Maturin and Peeler (2001). Five g of mushroom sample was placed in a sterile stomacher bag and homogenized using a stomacher (Seward) in 45 ml buffered peptone water (Acumedia lab, UK) for 30 s. Tenfold serial dilutions were prepared, then appropriate dilutions (10^{-6}) were pour plated onto plate count agar (Acumedia lab, UK) and incubated at 35 °C for 48 h.

-Coliforms

Coliforms enumeration was carried out using a method described by Feng et al. (2002). Dilution 10^{-1} was prepared by homogenising 5 g of mushroom sample into 45 ml of buffered peptone water (Acumedia lab, UK) for 30 s. Tenfold serial dilutions were prepared, and appropriate dilutions (10^{-6}) were pour plated in onto Violet Red Bile agar. (Acumedia lab, UK). A second overlay was performed with the Violet Red Bile agar and plates were incubated at 35 °C for 48 h.

-Yeast and moulds

Five g of mushroom sample was homogenised into 45 ml of buffered peptone water for 30 s. Tenfold serial dilutions (10^{-1}-10^{-6}) were prepared and spread-plated in duplicates on Rose Bengal chloramphenicol agar (Acumedia lab, UK) plates. The plates were incubated at 25 ˚C for 5 days.

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-Qualitative detection of Salmonella

Detection of *Salmonella* was carried out by modifying AOAC Official methods of Analysis 995.20:2016 (AOAC International, 2016). Sterile lactose broth (45 ml) was added to 5 g of mushroom samples. The mixture was incubated overnight at 35 °C. After incubation, 0.1 ml of the incubated mixture was transferred to Rappaport-Vassiliadis medium (Scharlau lab, Spain) and incubated for 24 h at 42 °C. After culturing on selective enrichment media plates of Xylose Lysine Desoxycholate (XLD) agar (Scharlau lab, Spain), the plates were assessed for *Salmonella* after 24 h of incubation at 35 °C.

Mycotoxins analyses

Truffles (T_1, T_5, T_6, T_7, and T_8) were analysed for deoxynivalenol, fumonisin B_1, ochratoxin A, total aflatoxins, and zearalenone using Enzyme-Linked ImmunoSorbent Assay (ELISA) kits (Elabscience®, USA). Each kit was used for extraction and analysis, according to the manufacturer's instruction. The assays were performed in 96-microwell plates pre-coated with mycotoxin of interest. The Optical Density (OD) value of each well was determined by reading with a microplate reader (SpectraMax 190) set at the respective wavelength.

Heavy metal analysis and daily intake of metals

Heavy metals (Cu, Fe, Zn, Cd, Mn, Ni, Pb, Hg, Cr) analyses was carried out according to the method described by Giron (1973). The quantitative determination of elements was carried out using ICAP Series (ICP Spectrometer; Thermo Scientific, USA). Daily Intake of metal was calculated for the maximum detected level per analyzed metal following the equation and assumptions reported by Sarikurkcu et al. (2020). Briefly, the amount of mushroom consumed by an average adult person (assumed to be 30 g dried) was multiplied with the metal concentration in the dried mushroom. This mathematical product was then divided by the average body weight of the consumer, assumed to be 70 kg.

Statistical analyses

All tests were done in duplicates. Data was subjected to an analysis of variance (one-way ANOVA) and Duncan's least significant differences ($p<0.05$) test using SPSS Statistics Software, Version 25 (IBM, USA).

Results

Moisture content

The moisture contents of unwashed and washed truffles are given in Table 1. The moisture content of dried unwashed truffles ranged from 10.0 to 17.6%, whereas that of dried washed truffles was in the range of 7.8 and 17.5%. Fresh samples, unwashed, and washed truffles had moisture contents ranged from 70.0% to 72.5%.

Aerobic count, total coliforms, yeast, mould, Salmonella

The aerobic count, total coliforms, yeast, and mould counts in truffles are given in Table 2. Washing of truffles reduced the aerobic counts, but the counts did not differ significantly ($p≥0.05$) from those of the unwashed truffles except for sample T_8 where washing had significantly reduced the total aerobic counts ($p<0.05$). The total coliform counts were not significantly ($p≥0.05$) different between unwashed and washed truffles for samples T_2, T_3, T_4, T_6, and T_8, whereas for samples T_1 and T_7, the total coliform counts differed significantly ($p<0.05$) between washed and unwashed truffles. There was no detected yeast in the unwashed samples T_5, T_6 (fresh), T_7, and T_8. The yeast counts for these samples (T_5, T_7, and T_8) were significantly ($p<0.05$) higher in the washed counterparts. There was no *Salmonella* in each 10 g of truffle sample.

Mycotoxins

The results of mycotoxins in unwashed and washed truffles are presented in Table 3. Washing of truffles significantly ($p<0.05$) reduced the levels of deoxynivalenol, but the levels of fumonisin B_1 in unwashed and washed truffles had no significant ($p>0.05$) difference. The ochratoxin A levels differed significantly ($p<0.05$) between unwashed and washed samples in samples T_1 and T_5. Although the levels of ochratoxin A in samples T_5, T_6, T_7, and T_8 increased after the washing of truffles, this increase was not statistically significant ($p≥0.05$). Regarding the total aflatoxin and zearalenone, there were no significant ($p≥0.05$) differences between the levels analyzed in the unwashed and the washed truffles.

Heavy metals

The levels of studied heavy metals in unwashed and washed truffles are given in Table 4. Noteworthy is that Hg and Ni were detected in none of the truffle samples. The levels of Cd differed significantly ($p<0.05$) between the unwashed and the washed truffles in all samples except for samples T_1 and T_3. Essentially, washing of truffles reduced the levels of Cd in samples T_5, T_6, T_7, and T_8. The Cr levels in truffles did not differ significantly ($p≥0.05$) between unwashed and washed samples except for sample T_6 (fresh). The levels of Fe in unwashed and washed truffles differed significantly ($p<0.05$) in all the samples except for sample T_6 (fresh). The levels of Mn differed significantly ($p<0.05$) between washed and

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unwashed truffles in all samples except for samples T_5 and T_7. The levels of Zn differed significantly ($p<0.05$) between unwashed and washed truffles in all truffle samples except for truffle sample T_2, T_6, and T_6 (fresh). Based on the highest levels determined for each heavy metal in this study, the maximum daily metal intakes (μg/kg body weight day or one serving per day) were calculated. The maximum daily intakes for Cd, Cr, Fe, Mn, and Zn were 0.17, 7.14, 320.02, 4.10, and 65.67, respectively.

Figure 1: Batches of truffles sold at the open market in Namibia

Table 1: Moisture (%) content of Kalahari truffles sold in Namibia

Sample	Unwashed	Washed
T_1 (dried)	12.5^{bc} ±3.5	17.5^{a} ±3.5
T_2 (dried)	12.5^{bc}±3.5	13.95^{bc}±0.5
T_3 (dried)	10.0^{bc}± 0.0	12.05^{bc}±4.2
T_4 (dried)	17.6^{b}±4.6	15.4^{bc}±0.6
T_5 (dried)	10.0^{bc} ±0.0	10.0^{bc}±0.0
T_6 (dried)	15.0^{bc}±7.1	7.25^{c}±3.9
T_6 (fresh)	72.5^{a}±3.5	70.0^{a}±7.1
T_7 (dried)	12.5^{bc}±3.5	15.0^{bc}±2.1
T_8 (dried)	14.0^{bc}±0.5	7.8^{c}±3.1

T1-T8=Truffles from different vendors
Values are means of two replicates ±standard deviation
Values with different superscript letter (^{abc}) in a row differ significantly ($p<0.05$)

Table 2: Aerobic count, total coliforms, yeasts and mould (Colony Forming Unit (CFU)/g) in Kalahari truffles sold in Namibia

Sample	Aerobic count		Total coliforms		Yeast		Mould	
	Unwashed	Washed	Unwashed	Washed	Unwashed	Washed	Unwashed	Washed
T_1	6.4^{ab} ±0.0	5.5^{bc} ±0.0	6.2^{a} ±0.0	3.9^{bcd} ±0.1	7.2^{a} ±0.1	5.7^{bcd} ±0.5	5.8^{ab} ±0.7	4.7^{bcde} ±0.5
T_2	5.2^{bcd} ±0.5	4.6^{cd} ±1.2	4.5^{abcd} ±0.6	2.7^{cd} ±0.0	4.7^{def} ±0.8	4.5^{def} ±0.0	4.7^{bcde} ±0.2	3.7^{def} ±0.5
T_3	4.8^{cd} ±0.2	4.5^{cd} ±0.1	5.0^{ab} ±1.6	4.4^{abcd}±0.5	6.6^{ab} ±0.03	5.0^{cde} ±0.7	5.4^{bc} ±0.1	4.6^{bcde} ±0.4
T_4	5.5^{bc} ±0.0	4.9^{cd} ±0.7	3.4^{bcd} ±0.6	4.5^{abc} ±0.6	4.9^{cdef} ±1.5	5.3^{bcd} ±0.0	4.4^{cde} ±1.7	5.2^{bc} ±0.3
T_5	4.8^{cd} ±0.0	4.6^{cd} ±0.8	4.3^{abcd} ±0.7	ND	ND	3.5^{f} ±0.9	5.0^{bcd} ±0.6	4.7^{bcde} ±0.7
T_6	4.9^{cd} ±0.4	4.5^{cd} ±0.2	4.1^{abcd} ±1.1	2.4^{d}±0.0	3.7^{ef} ±0.3	4.9^{cdef} ±1.0	4.3^{cde} ±0.0	3.6^{ef} ±0.2
T_6 (fresh)	4.6^{cd} ±1.2	6.3^{ab} ±0.1	ND*	ND	ND	ND	2.9^{f} ±0.1	3.6^{ef} ±0.2
T_7	6.2^{ab} ±0.2	5.2^{bcd} ±0.5	5.1^{ab} ±0.0	2.5^{d}±2.0	ND	6.3^{abc} ±0.6	6.9^{a} ±0.5	5.5^{bc} ±0.7
T_8	7.3^{a} ±0.1	4.0^{d} ±0.0	3.6^{bcd} ±0.2	4.3^{abcd} ±1.0	ND	6.4^{ab} ±0.8	4.8^{bcde} ±0.1	4.8^{bcde} ±0.0

T_1-T_8 = Truffles from different vendors
Values are mean of two replicates ±Standard deviation,
Values with different letter superscript in columns (^{abc}) are significantly ($p<0.05$) different from each other.
*ND: Not Detected

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Table 3: Mycotoxins (µg/kg) in Kalahari truffles sold in Namibia

Sample	Deoxynivalenol		Fumonisin B_1		Ochratoxin A		Total aflatoxin		Zearalenone	
	Unwashed	Washed	Unwashed	Washed	Unwashed	Washed	Unwashed	Washed	Unwashed	Washed
T_1	25462.2^{c} ±109	26113.7^{a} ±30	17.4^{b} ±6.5	65.3^{ab} ±55.7	48.5^{a} ±9.2	0.1^{c} ±0.1	26.5^{a} ±0.4	27.5^{a} ±1.3	59.0^{b} ±4.0	9680.0^{a} ±9438.0
T_5	25887.7^{b} ±167	25130.3^{d} ±98	142.1^{a} ±115.6	23.9^{ab}±2.4	1.7^{c} ±2.2	19.1^{b} ±0.7	27.3^{a} ±1.1	26.5^{a} ±0.3	117.0^{b} ±10.0	63.0^{b} ±15.0
T_6	25933.3^{a} ±55	25556.2^{c} ±58	86.1^{ab} ±66	35.8^{ab}±27.7	0.8^{c} ±0.1	4.0^{c} ±2.2	26.6^{a} ±0.2	26.7^{a} ±0.1	89.0^{b} ±3.0	59.0^{b} ±9.0
T_7	24965.7^{d} ±101	25596.2^{c} ±95	73.0^{ab} ±21.7	18.1^{b}±4.0	0.6^{c} ±0.7	3.6^{c} ±2.0	27.7^{a} ±0.3	26.5^{a} ±0.0	271.0^{b} ±109.0	50.0^{b} ±5.0
T_8	25882.2^{b} ±53	25552.3^{c} ±73	140.4^{ab} ±13.1	49.2^{ab}±48.9	0.2^{c} ±0.2	3.2^{c} ±3.1	26.8^{a} ±0.1	26.3^{a} ±0.3	99.0^{b} ±18.0	45.0^{b} ±3.0

T1, T5, T6, T7, T8=Truffles from different vendors.
Values are means of two replicates ±Standard deviation, Values with different letter superscript (^{abc}) in columns per mycotoxin are significantly (p<0.05) different

Table 4: Heavy metals (mg/kg) in Kalahari truffles sold in Namibia

Sample	Cadmium (Ca)		Chromium (Cr)		Iron (Fe)		Manganese (Mn)		Zinc (Zn)	
	Unwashed	Washed	Unwashed	Washed	Unwashed	Washed	Unwashed	Washed	Unwashed	Washed
T_1	0.14^{f}± 0.01	0.26^{def}±0.06	10.1^{de} ±0.5	16.67^{a} ±1.3	521.04^{b}± 36.4	746.72^{a}±40.0	5.61^{c} ±0.5	6.10^{b}±0.1	153.24^{a}±9.2	103.61^{c} ±3.0
T_2	0.81^{b} ± 0.2	0.34^{de}± 0.1	9.31^{def} ±1.1	5.75^{g}±0.3	300.03^{e} ±27.4	166.64^{g}± 2.5	3.15^{f}±0.3	2.67^{g} ±0.01	67.93^{e} ±3.3	65.02^{e}±1.9
T_3	0.28^{def}± 0.09	0.21^{ef}± 0.02	10.74^{cd}±0.3	14.07^{b} ± 0.4	434.34^{c}± 15.5	531.44^{b}± 15.2	4.75^{d}± 0.2	5.57^{c}±0.03	135.17^{b}±7.1	51.98^{gh} ± 0.3
T_4	0.39^{cd}±0.0	0.16^{f}± 0.08	14.50^{b}±0.5	2.23^{h}± 0.2	404.30^{cd}± 17.8	120.03^{h}± 0.01	3.84^{e}± 0.1	1.50^{ij}±0.01	49.56^{gh} ±2.8	61.79^{ef}± 1.7
T_5	0.27^{def}± 0.0	ND	12.14^{c}±1.3	2.57^{h}± 0.05	259.21^{f}± 4.1	147.62^{gh} ±15.1	2.79^{fg}±0.03	2.76^{fg}±0.04	56.13^{fg}± 0.6	46.26^{h}±0.2
T_6	0.25^{def} ± 0.02	ND	5.66^{g} ± 0.5	8.16^{f}±1.6	108.96^{h}± 22.5	377.33^{d}±30.2	1.77^{hi} ±0.3	2.84^{fg}±0.3	45.33^{h} ± 2.9	46.43^{h}±3.5
T_6 (fresh)	0.16^{ef} ± 0.06	ND	1.13^{hi}±0.05	0.28^{i}± 0.03	42.66^{i}± 3.7	55.19^{i}± 1.8	0.57^{k}±0.03	0.66^{k} ±0.0	11.70^{i}±1.0	8.25^{i}± 0.2
T_7	0.53^{c}±0.07	ND	4.59^{g} ±0.3	2.09^{h}± 0.3	241.34^{f} ± 11.1	117.18^{h}±2.2	1.98^{h}±0.1	1.22^{j} ±0.1	85.57^{d} ±4.8	65.09^{e}± 1.3
T_8	0.27^{def}± 0.09	1.35^{a}± 0.06	8.62^{ef}± 0.6	10.65^{cd}±0.8	366.67^{d}±14.5	444.27^{c}± 12.9	5.20^{c}±0.2	9.56^{a}±0.01	52.61^{gh}± 1.3	136.63^{b}± 0.4

T1-T8=Truffles from different vendors.
Values are means of two replicates ± standard deviation
Values with different superscript letters in rows per heavy metal differ significantly (p<0.05); ND: Not Detected

Discussion

The microbiological, mycotoxins, and selected metals were assessed in Kalahari truffles to generate baseline information on the quality and safety of these wild manifested food resources in Namibia. The fresh truffles moisture content was above the 68% reported by Wahiba et al. (2016) in truffles from Iran, but similar or lower than 73-77% moisture values reported by Yousif et al. (2020) and Behzadi et al. (2021) in truffles from Iraq and Iran, respectively. As can be seen in Figure 1, the truffles are kept in an open air at the open markets until they are sold. This exposure allows for evaporation and thus the relatively low moisture content found in this study than what is commonly reported for fresh truffles.

The aerobic counts detected in both unwashed and washed truffle samples in this study were less than those found in other truffle species in Spain (Rivera et al. (2010), Italy (Saltarelli et al., 2008), and Spain (Phong et al., 2022). The differences in the microbial counts could be linked to the heterogeneity of the sample, the harvesting season, mechanical damage or internal parasitisation (Rivera et al., 2010). The directive 2004/24/EC of the European Commission (2004) states that the acceptable total aerobic count limit is <5.7 log CFU/g. So, all the truffle samples of present study had counts below the acceptable limit except for sample T6 (fresh) that had counts above the acceptable limits based on European Commission (2004). Overall, the results indicated that the majority of truffle samples had suitable sanitary quality.

The total coliforms results were similar to those reported by Cirlincione et al. (2021) on black summer truffles in Italy. The unwashed truffle (T_1, T_2, T_3, and T_8) and the washed T_4 samples had higher total coliform counts than the total coliforms counts (4.0 log CFU/g) that Reale et al. (2009) detected on fresh black truffles in Italy. These differences could be attributed to the type of species and the level of soil contamination from which the truffles were harvested. The presence of coliforms in foodstuff may indicate poor hygienic quality of truffles. This suggests that these truffles should be subjected to thorough cleaning and processing such as cooking. The acceptable limit for total coliforms based on European Commission (2004; directive 2004/24/EC) is <1 CFU/g. Based on these recommendations, only samples T_6 (fresh) and T_5 (washed) had total coliform counts below the acceptable limit.

The yeast counts were in the same range as those reported by Cirlincione et al. (2021) on black summer truffles in Italy. They were also similar or higher than the 4-5.5 log CFU/g reported in truffles from Spain by Phong et al. (2022) and were higher than the 3.4 log CFU/g yeast counts that Rivera et al. (2010) detected in dried *Tuber aestivum* and *Tuber melanosporum* truffles samples that were also from Spain. The presence of yeast in food products is not a hazard to health, but consumption of yeast contaminated food could lead to allergic reactions and food spoilage. The acceptable limit of yeast and mould count based on European Commission (2004;

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directive 2004/24/EC) is <log 3.7 CFU/g. Irrespective of washing of truffles, majority of the samples had yeast counts above the acceptable limits of the European Commission (2004; directive 2004/24/EC). This could indicate a shorter shelf life of truffles and thus, further preservation methods may be necessary.

Washing of truffles generally reduced mould counts. However, the mould counts between unwashed and washed truffles did not generally differ significantly. For most of the samples irrespective of washing, mould counts were similar or higher than the values reported by Cirlincione et al. (2021) on black summer truffles in Italy and by Tejedor-Calvo et al. (2020) on fresh truffles in Spain. Furthermore, the mould values in this study were higher than the 3.7 log CFU/g that Rivera et al. (2011) detected in fresh *T. aestivum* truffles from Spain. Regardless of washing of truffles, only three samples had mould counts below the acceptable limit of <3.7 log CFU/g based on the European Commission (2004; directive 2004/24/EC). This study did not detect *Salmonella*, which is in agreement with the findings of Reale et al. (2009) in fresh black truffles in Italy and those of Phong et al. (2022) in truffles from Spain.

There is little published studies in the literature regarding evaluation of mycotoxins in truffles. The discussion on mycotoxins results is therefore limited to comparison with other foods where a particular mycotoxin had been investigated and/or relating the findings to existing regulations such as those of the Food and Drug Administration (FDA). Although washing of truffles had significantly reduced the levels of deoxynivalenol, all the truffle samples had deoxynivalenol levels above the 500 µg/kg in foodstuffs. Since mycotoxins are stable chemicals, decontamination of truffles should be carried out to reduce the levels of deoxynivalenol to safe levels.

Nevertheless, fumonisin B_1 has been reported in some tubers. Amri and Lenoi (2016) reported the occurrence of fumonisin B_1 (12.34 to 267.86 µg/kg) in dried sweet potato chips. The United States FDA has the regulatory levels for fumonisins (2,000-3,000 µg/kg) in foodstuffs (FAO, 2004). Based on FDA regulations, the levels of fumonisin B_1 detected in all the truffle samples irrespective of washing were below the maximum allowable limits. This indicates that truffles were of good quality and safe for consumption with regards to fumonisin $B_{1.}$ The advisory limits of ochratoxin A in foodstuffs for European Union is set at 5 µg/kg (FAO, 1997). Based on the European Union regulations, only samples T_1 (unwashed) and T_5 (washed) had levels above the advisory limits, while 80% of the samples had ochratoxin A levels within the acceptable limits.

Washing of truffles had no significant effect on total aflatoxin levels in truffles. Even though occurrence of aflatoxin in truffles has not been reported in literature, Jonathan and Esho (2010) detected aflatoxin B_1 (1.93 µg/kg to 4.21 µg/kg) in dried and stored Nigerian Oyster mushrooms. FDA established regulatory limits of 20 µg/kg for total aflatoxin in foodstuffs (FAO, 2004). Based on these regulations, all unwashed and washed truffles investigated in this study had total aflatoxin levels above the allowable limits. Deoxynivalenol and aflatoxin occurrence of up to 99% and 66%, respectively is reported in grains and grain-based food products. Also, other processing methods such as fermentation and heat treatments can significantly reduce the mycotoxin levels (Sarmast et al., 2021).

As stated, the occurrence of zearalenone in truffles has not been reported in literature. However, zearalenone occurrence in medicinal dried rhizomes (*Acorus calamus*, *Bergenia ciliata*, *Curcuma longa*, *Zingiber officinale*) and root tubers (*Pueraria tuberosa*) has been reported by Koul and Sumbali (2008) and were in the range of 520 to 14,510 µg/kg roots and tubers, which like truffles grow underground. The levels of zearalenone in truffles were less or similar to the levels detected by Koul and Sumbali (2008) in dried rhizomes and root tubers collected from India. Thailand established the maximum limits for zearalenone (30-1,000 µg/kg) in foodstuffs (Anukul et al., 2013). Based on zearalenone regulations for Thailand, almost all the truffle samples had zearalenone levels were within the acceptable limit. Ezekiel et al. (2013) did not detect any mycotoxin in the dried mushrooms from Nigeria. This study gives a preliminary basis upon which confirmation is warranted using advanced techniques such as Liquid Chromatography-Mass Spectrometry (LC-MS) to identify and quantify the specific mycotoxins and microorganisms.

The absence of Hg and Ni could be an indication that the fields from which the truffles were harvested are not contaminated with Hg and Ni. Hg was however found in some mushrooms. Fang et al. (2014) detected 0.02 mg/kg in dry mushrooms. Presence of low levels of Hg in foodstuffs can seemingly be allowed. The permissible levels for Hg in food is 0.6 mg/kg as per Codex Alimentarius Commission (2011) standard.

Except for three truffle samples, all others had lower levels of Cd that the 0.404 mg/kg reported by Xu et al. (2019) in truffles in China. Similarly, the levels of Cd in all truffles were lower than the 54.2 mg/kg Cd level that Sarikurkcu et al. (2011) detected in wild edible mushrooms from Soguksu National Park in Ankara in Turkey. They were also lower than the Cd levels of up to 148 mg/kg reported by Michelot et al. (1999) in most mushrooms' species collected from Latin America (French Guyana, Colombia, Costa Rica). These differences can be attributed to levels of Cd contamination of the fields where truffles were harvested. The permissible limits for Cd in food samples is 1.0 mg/kg (European Commission

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(2008) (directive 2008/629/EC). Based on this, all truffle samples in this study had Cd levels within permissible levels irrespective of washing.

The levels of Cr determined in this study are similar or lower that those reported by Sarikurkcu et al. (2011), who reported a range from not detected to 21.6 mg/kg in mushrooms originated from Soguksu National Park in Ankara in Turkey. They were also within the range of 4-22 mg/kg Cr amounts in truffles collected from Iraq reported by Qazmooz et al. (2020). On the other hand, they were higher than the 0.036-0.05 mg/kg Cr levels in truffles from Turkey reported by Akyüz and Kirbag (2018). The Fe levels were within the 10.4-4,900 mg/kg range reported by Michelot et al. (1999) in the majority of mushrooms species collected from French Guyana, Colombia, and Costa Rica. Moreover, this study Fe results were similar or higher than the Fe levels (400-500 mg/kg) detected in *Amanita rubescens* mushrooms from Poland by Rudawska and Leski (2005a, 2005b). Sarikurkcu et al. (2020) reported some mushroom samples from Turkey with higher Fe levels up to 1,580 mg/kg in mushrooms, which is more than double the levels found in this study.

Mn levels detected in this study were lower than the 10-77 mg/kg that Rudawska and Leski (2005a, 2005b) reported in mushroom species collected from the wild in Turkey. Michelot et al. (1999) found a wide range (4.1-400 mg/kg) of Mn amounts in many mushrooms species collected from French Guyana, Colombia, and Costa Rica, which encompasses the results of this study. The Mn results were, however, higher than the 0.02-0.112 mg/kg Mn levels in truffles from Turkey reported by Akyüz and Kirbag (2018).

Falandysz et al. (2001) detected Zn that was as high as 460 mg/kg in dried mushrooms from Poland. This was much higher than this study's findings. Similarly, the Zn results of this study were lower or within the 23.9-369 mg/kg of Zn found in mushrooms species collected from French Guyana, Colombia, and Costa Rica (Michelot et al., 1999). Some samples in this study had higher amounts of Zn than the 55.6-57.3 mg/kg that was determined in truffles from Iran (Behzadi et al., 2021). Zn is apparently an antagonist of other metals such as Cd, Pb, and Ni. Thus its presence in some mushrooms can potentially reduce the risks associated with other toxic metals at high concentrations (Codex Alimentarius Commission, 1995). The metals (Cd, Fe, Zn, and Mn) intake levels results, based on the maximum amounts quantified in this study, showed that all the truffle samples were within the respective metal consumption safety range, except for one sample. This is based on the reference daily intakes (µg/kg/day) of 0.5 for Cd, 300 for Fe, and Zn and 140 for Mn (Sarikurkcu et al., 2020). Using 70 kg as the average weight of an adult, the upper recommended daily intake reported in Marini et al. (2021) for Cd, Cr, and Zn were 0.36, 4.29 and 357.14 µg/kg/day, respectively. The values for Cr in truffles used in this study were higher than the recommended daily intake. A survey on the human intake of Cd across European countries through food consumption found a higher intake than the reference daily intakes (EFSA, 2011). The upper daily Mn intake by an adult person recommended by the EFSA NDA (2013) is 42.86 µg/kg/day. This was over 10 times more than what the studied truffles can deliver based on Sarikurkcu et al. (2020) formula. These results can be the basis to build and confirm the understanding of elemental composition of Kalahari truffles.

Conclusion

The aerobic counts in 87.5% of the truffle samples were within the acceptable limits. Washing reduced the total coliforms, yeast, and mould counts in over 70% of all the truffle samples. *Salmonella,* Hg, and Ni were not detected in any of the truffle samples. Deoxynivalenol, fumonisin B_1, Ochratoxin A, total aflatoxin, and zearalenone were detected and further advanced quantification is warranted. Almost all the truffle samples had Cd, Cr, Fe, and Mn levels within the daily metal intake levels by an average adult regular consumer. The analyzed microorganisms, mycotoxins, and heavy metals in underprized Kalahari truffles may impair the safety, shelf life, and human health. Thus, they should be subjected to appropriate processing before consumption.

Author contributions

T.A.H., W.E., K.K.M.N., and N.P.K. designed the experiment; W.E., K.K.M.N., and L.I. adapted the methods; T.A.H. did experimental work, analyzed data, and wrote the manuscript; W.E., K.K.M.N., N.P.K., and L.I. facilitated the resources, edited, and reviewed the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors have no competing interests.

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