A 500-year-old medicine container discovered near Misgund, Eastern Cape, South Africa: Residue characterisation by GC-MS

The chance discovery of a 500-year-old cattle-horn container in a painted rock shelter on the farm La vie D’Antan in the Eastern Cape Province of South Africa sheds new light on the antiquity of traditional medicines in the region. We report the micro-residue and GC-MS results of the solidified substance found inside the horn container. Several plant-based medicinal compounds were tentatively identified, of which mono-methyl inositol and lupeol are the most prevalent. Based on pharmacobotanical studies, we suggest the most probable ailments the medicine would have been used to treat and propose the most likely plants from which the ingredients were sourced. Apart from the rock art, whose contemporaneity has not been established, there is no associated archaeology from which to draw specific cultural associations. Although people clearly have been aware of the medicinal properties of plants for at least the last 200 000 years, this is, to our knowledge, the oldest evidence from southern Africa of a bespoke container that has been used to store multiple combined ingredients of medicinal application. The age of the contents of the horn container, however, could not be independently established, leaving open the possibility that the medicinal container and its contents may not be contemporaneous.

Significance:
- We present the oldest medicine container yet found in southern Africa combining two or more plant ingredients.
- The findings add to our knowledge of traditional Khoisan medicines and the antiquity of this traditional knowledge system.

Introduction

The presence of organic residues on archaeological artefacts invites curiosity. Trace amounts of residue recovered from stone and bone tools in ancient archaeological deposits have been shown through chemical analysis to have been part of adhesive and poison applications. In most cases, plant exudates have formed the basis of these applicable substances. The combustion of certain plants, chosen for their insecticidal properties, seems to have been deliberately carried out as a means to fumigate living areas from at least 200 ka. The understanding and harnessing of the chemical and pharmacological properties of plants and the ability to combine these into novel and complex recipes allowed humankind to successfully adapt to diverse environments. Whereas there is now a wide body of literature that explores some of these poison and adhesive recipes and their antiquity in southern Africa, the same is not true for traditional medicines.

Traditional medicine continues to play an important role in much of Africa as a primary health service. Traditional medicine is highly adaptive and does not shy away from incorporating alien plant species or foreign pharmacopeia. The South African trade in medicinal plants has been valued at ZAR270 million annually, with the Eastern Cape Province alone accounting for 166 of the 700 plant species traded nationally. However, only 33 plant species traded in the Eastern Cape are pharmacologically efficacious, with the others being used to treat supernatural afflictions. Indeed, one of the principal functions of Khoisan traditional healers was to treat supernatural bewitchment. Medicine and culture were intimately entwined.

Most of our knowledge about traditional medicines is derived from early travellers and ethnographic accounts, with older medicinal remedies being largely inferred from these. In the absence of archaeological evidence, it is difficult to know how far back in time these traditional medicines date, or indeed how traditional medicines may have changed over time. We do not know whether the use of certain plants has a greater antiquity than that of others, nor how pre-colonial medicines were administered, nor whether the plants that were used were always pharmacologically effective in the treatments for which they were administered. The presence of plants with medicinal properties in Middle Stone Age deposits suggests that people were aware of these properties, but gives no indication as to whether and to what extent different plant ingredients may have been combined to create applicative ointments or oral remedies.

A recent discovery in the Langkloof mountains of the Eastern Cape, South Africa, now provides the opportunity to learn about a possible medicinal ointment used by Khoi or San people 500 years ago. In 2020, a sealed cattle horn filled with an unknown substance was recovered from a farm that lies 40 km from the towns of Uniondale in the west and Plettenberg Bay in the south (Figure 1). Based on the orientation of the horn contents, this substance must have been in a liquid or gelatinous state when deposited, but had since hardened into a solid, tough, tacky mass. The substance is readily soluble in water, taking only a few minutes to reconstitute into a liquid state. In this paper, we present the results of a chemical analysis of the horn contents carried out using gas chromatography–mass spectrometry (GC-MS).
Background

The cattle-horn container was recovered from a small, painted rock shelter (33°44'14.06"S; 23°34'37.63"E) on a privately owned farm, called La vie D’Antan, in the Eastern Cape. The farm is located 8 km from the small hamlet of Misgund and within 10 km of where the 2000-year-old Kouga mummy was discovered by Johan Binneman in 1999. The rock shelter is set in the Enon Formation conglomerate and is approximately 3.5 m deep and 3 m high, containing shallow floor deposit, only a few centimetres thick. The horn was capped with a rawhide lid and wrapped in a bundle of Boophane disticha leaves and grass (Figure 1). The leaves and grass were secured to the horn by twisted plant fibre rope. Boophane leaves are known for their antiseptic properties and may be the reason the horn has preserved so well. The only visible damage to the horn is the activity of dermestid beetles. The parcel had been exposed through animal activity and was removed by a visitor to the site to prevent further damage to it. Apart from a few undecorated ceramic pot shards scattered across the surface, the shelter contains no other artefacts nor any evidence of long-term occupation. The landscape surrounding the shelter abounds in various Helichrysum and Senecio species. Both plants are edible and widely used to brew tea and other beverages.

The rock surface inside the overhang comprises a conglomerate of numerous sandstone boulders. Approximately 20–30 rock paintings in varying shades of red and yellow ochre-based paint occur across the width of the overhang. The rock art was probably made with brushes (except the handprints). The subject matter – human figures with hunting equipment, one clearly preserved eland in an alcove, a partially preserved antelope and indeterminate animals, as well as handprints – is typical of the hunter-gatherer (San) rock art in the area. The preservation of the paintings is mostly poor with a few clearer images. No age determinations have been made on the paintings. There are two other rock art sites within a couple of kilometres of this shelter (Figure 2).

Prior to the 18th century, the region was inhabited by San hunter-gatherers and Khoi pastoralists, the latter of whom owned large herds of cattle and sheep. By 1775, the Langkloof region was occupied predominantly by Dutch farmers and Inqua Khoi. The Khoi and the few remaining San hunter-gatherers were by this time mainly employed as client herdsmen on the Dutch farms. Although mixed San and Khoi descendants may have persisted in these mountains as fugitives and military absconders until as late as the 1880s, the last independent San bands with a Later Stone Age economy were probably dispersed by the 1760s. Not much is known about the last hunter-gatherers of the Langkloof. Stow alludes to them but defers description to a future book.
which he never wrote. Suffice it to say that the wider region contains ample evidence of Later Stone Age hunter-gatherer occupation spanning at least the last ten thousand years.37,31

The proximity of the La vie D’Antan rock shelter to the Kouga mummy site is intriguing. In 1999 the mummified remains of a 30–40-year-old man were found in the Kouga Mountains, which forms part of the Langkloof range. The remains were wrapped in Boophane disticha leaves and the last joint of the left little finger had been amputated, as was customary in some San societies.32 The mummified remains returned a date of 1930±20 BP and, based on the height of the skeleton, it is thought to have belonged to a San rather than a Xhosa individual.21,33 No cause of death was ascertained.

Horns are known to be used as medicine containers throughout Africa, although in the southern African context tortoise shell and ostrich eggshell are far more common for this purpose.34-36 Our knowledge of horn containers derives exclusively from the historical period. Several cattle-horn containers from the 19th century are preserved in museums around the world. Snuff containers made from decorated cattle horns collected in 1892 and attributed to the Shona and Sotho are housed in the British Museum.37 Two cattle-horn medicine containers, collected in 1871 from southern Tanzania and northern Zimbabwe, were donated to the Harvard Peabody Museum.38 A similar medicine horn collected between 1890 and 1930 from the Belgian Congo pops up on a JSTOR search of ethnographic collections in the UK, but no further information is available on this item. Certain San groups in the Kalahari and the Bembe in Zimbabwe used antelope horns as medicine containers. Duiker horns were favoured in the Kalahari to store medicine associated with witchcraft17, while the Bembe used different antelope horns to store different medicines, preferring duiker and bushbuck18. In neither case were special attributes attached to the horn container itself.

Methods
An initial micro-morphological assessment of the horn contents was carried out using a multi-capability Olympus BX51M light microscope under varying magnifications, ranging from 100x to 1000x. Micro-residues taken from dry scrapings of the horn contents were observed under reflected light following established analytical protocols.39-44 Scrapings were taken up to a depth of 5 mm from the solidified substance. Next, a tiny sample was augered from the centre of the solidified substance up to a depth of 20 mm. The outer layer was discarded to reduce the influence of contaminants and the remainder of the sample was weighed out in 60-µg amounts into 2-mL Eppendorf tubes, into which was added 600 µL high-purity mass spectrometry grade organic solvents for extraction. These solvents were methanol, acetone and isopropanol (SpS, Romil, Cambridge, UK). The samples were vortexed and incubated at room temperature for 5 days to dissolve. However, not all of the original material dissolved. The methanol extract was brown and the acetone and isopropanol extracts light yellow. Extracts were analysed on a GC-MS QP2010 system (Shimadzu, Kyoto, Japan) equipped with an electron impact ion source and a single quadrupole mass analyser. A volume of 2 µL of the extracts was injected in splitless mode. The capillary column was a Restek Rtx 5 MS, diphenyl dimethyl polysiloxane column (30 m x 0.25 mm; 0.25 µm thickness) (Leco Africa, Kempton Park, South Africa). Analytes present in the extracts were separated with temperature gradient programming and detected with mass spectrometry. Chromatographic conditions were as follows: injection temperature: 250 °C; oven temperature: 50 °C, hold time 3 min; increase to 300 °C and hold time 5 min, increased at a rate of 25 °C/min; carrier gas: helium, flow rate 1.05 mL/min. The MS conditions were as follows: solvent cut of 2 min; detector voltage: 1.39 kV; ion source temp: 200 °C; interface temp: 250 °C. The MS data were acquired in scan mode over a 50–350 m/z range with a mass spectral acquisition rate of 1111 u/s. Samples were analysed in triplicate and method blanks with methanol, acetone and isopropanol were included. Solvent blank chromatograms are displayed in Figure 3.

Data acquisition was by means of the GC-MS Solutions software (Shimadzu, Kyoto, Japan). Tentative organic compound identifications (annotations) were acquired by matching mass spectral data (mass quality cut-off criterion ≥80% for the main analytes) to the Wiley Registry / NIST library, ver. 6 (https://sciencesolutions.wiley.com; John Wiley & Sons, Hoboken, NJ, USA) as well as the Flavour & Fragrance Natural & Synthetic Compounds GC-MS library (FFNSC 2, Shimadzu, Kyoto, Japan). Chromatograms of the solvent-specific extracts are shown in Figure 4.

Results
A sample of the horn was removed for radiocarbon dating at iThemba Laboratories in Johannesburg. The sample returned a carbon age of 380±30 BP (IT-C-3711), which calibrates using the SHCal20 curve16 to AD 1461–1630. At this period, the only two groups known archaeologically to be living in the area were hunter-gatherers and Khoi herders. We attempted to date a sample of the contents of the horn to establish contemporaneity. Unfortunately, two attempts to date this sample proved unsuccessful due to insufficient carbon content.
Figure 4: Gas chromatographic – mass spectrometric (GC-MS) profiles (total ion chromatograms) of analytes present in methanol, acetone, and isopropanol extracts of the substance in the horn container. The y-axis indicates the MS detector response (signal intensity counts). Refer to Table 1 for description of the numbered peaks.

The micro-morphological residue analysis revealed many insectan remains and plant tissues (Figure 5A–K). The insectan residues were present only in the outer layers of the horn contents, which were exposed through destruction of the horn by dermestid beetles. Samples taken from deeper inside the horn contents revealed no insect or plant tissue, suggesting that these are contaminant residues and not purposely added ingredients. The insect remains are too fragmentary to identify to species, but most could have come from dermestid beetles. The presence of Lepidoptera wing scales, however, suggests that the horn was exposed for a period at some point prior to its removal from the site. The main content of the horn appears as a shiny, brown crystalline substance, which dissolves easily in water (Figure 5L–O). When dissolved, the solution emits a slight liquorice odour. There were no signs of any plant, animal or insect cellular structures in the solution.

Table 1 presents the results of the GC-MS analysis. The main compounds detected were mono-methyl inositol (or isomers of pinitol) and lupeol. Table 1 also presents the percentages of these substances relative to the whole sample per solution (i.e. the percentage relative peak area of the total ion chromatogram), and the confidence levels expressed as the percentage match to the mass spectral library. The vast majority of the substance in the horn container consisted of mono-methyl inositol isomers (MoMe inositol). Trace compounds detected consisted of di- and tri-terpenes, a sterol derivative and fatty acid methyl esters (Table 1). Interestingly, no volatile aromatic compounds that might account for the faint liquorice odour were identified in the extracts by GC-MS.

Discussion

All of the tentatively identified organic compounds have medicinal applications. Inositol is a natural polysaccharide sugar found in a wide range of plants, including legumes, citrus fruit and various seeds. It is synthesised by the cell and used in the production of plasma lipo-proteins to aid cell growth. It has many medicinal applications including the control of diabetes, the treatment of high cholesterol, bronchopulmonary dysplasia and various mood disorders (it stimulates the production of serotonin and dopamine), and the amelioration of symptoms associated with polycystic ovarian syndrome. Inositol is considered a pharmacologically safe compound as it cannot be overdosed on, and there are few reported side effects from prolonged use, apart from nausea, headaches, and hypoglycaemia in extreme cases.

Mono-methyl inositol and its isomer pinitol occur in several traditional medicinal plants found in the general study area, including Sutherlandia frutescens, Cyclopia intermedia, Lotonius laxa and Clitoria ternatea. S. frutescens has strong antioxidant properties and was used by the Khoi for washing wounds and treating fevers and eye infections. It is also taken as a tonic to boost the immune system and to alleviate symptoms of rheumatism and pulmonary ailments. Several Euphorbia species containing inositol are used to treat similar conditions. C. ternatea contains a high concentration of mono-methyl inositol (39%). Although it is found in the study region, its recorded medicinal use comes from Asia where it is used to treat sexually transmitted diseases and as an anti-anxiety medication.

Lupeol is a pentacyclic triterpenoid occurring in a wide variety of fruits and vegetables, and is a common constituent of balsams, resins and waxes.
Lupeol also occurs in several medicinal plants and is known for its anti-inflammatory and antimicrobial properties. In addition, it is increasingly being used in cancer treatments and antimalarial research owing to its antioxidant properties and the fact that it is pharmacologically safe, with little risk of ingestive overdosing.

Lupeol occurs in several plants used medicinally in South Africa and elsewhere. Relevant to our study region it occurs in *Ficus cordata*, *Asteracantha longifolia* and several different Euphorbia species. *A. longifolia* is used by the Pedi (and in India, where it also occurs) mainly to treat rheumatism and urinary tract infections, but also for gout and jaundice and as an aphrodisiac.

Kaurenoic acid is a diterpenoid present in the resin and sap of several plants and is highly effective against Gram-positive bacteria such as listeria, staphylococcus and streptococcus. It also exhibits protective activity against liver damage.

Kaurenoic acid is present in several plants in the study area, most commonly in *Arctopus sp.*, *Alepidea sp.* and *Aster bakeranus*. The former was first recorded in 1770 as a Khoi medicine to treat gout, various infections and respiratory ailments. *A. bakeranus* is similarly thought to have been used for medicinal purposes by the Khoi, and is snuffed or ingested as treatment for venereal diseases and urinary tract infections, although among the Zulu it is additionally used to treat chronic coughs and intestinal complaints.

Cyclolanostenol acetate or lanosterol is a tetracyclic triterpenoid that can be synthesised by animals and dicotyledonous plants, where it is the precursor to the synthesis of all plant steroids. *Rafnia amplexicaulis*, present in the study area and containing lanosterol, is reported to have been used as a tea to treat pulmonary issues, although more recent GC-MS characterisation of 500-year-old medicine containers...

### Table 1: GC-MS analysis

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Spectral peaks</th>
<th>Retention time (Rt)</th>
<th>Molecular weight (g/mol)</th>
<th>Molecular formula</th>
<th>Tentative compound name (identifier numbers)</th>
<th>Spectral match (%)</th>
<th>Relative peak area (%)a</th>
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<td><strong>Methanol</strong></td>
<td>1</td>
<td>7.81</td>
<td>110.15</td>
<td>C7H10O</td>
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<td></td>
<td>2</td>
<td>10.02</td>
<td>192.17</td>
<td>C7H12O6</td>
<td>Quinic acid (CAS: 77-95-2; NIST: 1051817; PubChem: 6508)</td>
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<td>t</td>
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<tr>
<td></td>
<td>3</td>
<td>10.20</td>
<td>194.18</td>
<td>C7H14O6</td>
<td>Mono-methyl (MOME) Inositol isomer I</td>
<td>89</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>10.72</td>
<td>194.18</td>
<td>C7H14O6</td>
<td>MOME Inositol isomer II (e.g. D-pinitol) (CAS: 484-68-4; NIST: 1051878; PubChem: 164619)</td>
<td>88</td>
<td>69</td>
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<tr>
<td></td>
<td>5</td>
<td>11.08</td>
<td>270.5</td>
<td>C6H12O6</td>
<td>Hexadecanoic acid, methyl ester (CAS: 112-39-0; NIST: 1211052; PubChem CID: 8181)</td>
<td>82</td>
<td>t</td>
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<td></td>
<td>6</td>
<td>11.78</td>
<td>296.5</td>
<td>C19H36O2</td>
<td>7-Octadecenoic acid, methyl ester (CAS: 57396-98-2; NIST: 43709; PubChem CID: 5364440)</td>
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<td>t</td>
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<tr>
<td></td>
<td>7</td>
<td>11.84</td>
<td>328.5</td>
<td>C30H50O</td>
<td>Spiro[androst-5-ene-17.1-cyclobutan]-2-one, 3-hydroxy- (CAS: 60534-16-9; NIST: 55793; PubChem: 534435)</td>
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<td><strong>Acetone</strong></td>
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<td>116.1</td>
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<td>Diacete alcohol (2-Pentanone, 4-Hydroxy-4-methyl-) (CAS: 123-42-2; NIST: 159868; PubChem: 31256)</td>
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<td>330.5</td>
<td>C22H34O2</td>
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<td>426.7</td>
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<td>Lupeol (CAS: 545-47-1; NIST: 1053218; PubChem: 259846)</td>
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<td>C7H14O6</td>
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<td>426.7</td>
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<td>C30H50O</td>
<td>Lupeol isomer II</td>
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*aExpressed as percentage relative peak areas of the total ion chromatogram, t = trace amount*

research states that it has no medicinal value but is used as a substitute for liquorice as it has a similar taste. The three decanoic acids that were tentatively identified in the sample also have medicinal properties and are found in several plants in the study area. Hexadecane and octadecane acid both have antifungal, antibacterial and antioxidant properties. Hexadecanoic acid, together with lupeol, is used in antimarial treatments. Its methyl palmitate form, hexadecane is found in several species of Ciuta and Cyclopia and is taken orally for the treatment of colic and hepatitis, and applied topically as an anti-inflammatory and a carciade. While the tentatively identified organic compounds are present in a wide variety of plant genera, we can narrow down the likely plants that may have been used to constitute the substance in the cow-horn container by looking at which plants present in the area contain two or more of the tentatively identified compounds. Three species stand out. Corbichonia decumbens comprises 75% mono methyl inositol, 17% hexadecanoic acid and 16% octadecanoic acid. The plant is liquidised and drunk by the Zulus as an emetic. Glycyrrhiza glabra likewise contains high quantities of mono-methyl inositol (28%), octadecanoic acid (3.4%) and hexadecanoic acid (4.9%). Although thought to have been introduced by early European settlers it is used as a cough remedy in north Africa and as an anti-inflammatory in southern Africa. Another plant that occurs in the broader region and which contains several of the tentatively identified compounds (namely mono-methyl inositol, hexadecanoic acid and octadecanoic acid) is Macrotyla uniflorum. Although it is not recorded as a medicinal plant in southern Africa, it is used in India as an antioxidant and to treat insulin resistance. Another promising plant candidate is Milkania sp., some species of which contain lupeol and kaurenic acid. Certain Milkania sp. are used to treat snake bites and as a remedy for venereal sores.

Conclusion
Based on our analysis, the horn contains a medicinal substance, composed of at least two plant ingredients. We cannot know for certain which plant species were used, but we can narrow down the list of potential candidates. The main compounds present in the container, mono-methyl inositol and lupeol, have a wide range of recorded medicinal applications, including the control of blood sugar and cholesterol levels, treatment of fevers, inflammation (including pulmonary) and urinary tract infections, and may be applied topically to treat infections. Indeed, rubbing ointment into subdermal cuts is one of the ways the San are known to have treated skin infections, and may be applied topically to treat infections. While the tentatively identified compounds are pharmacologically safe compounds, meaning they can be ingested without risk of overdose. Both compounds stimulate the production of dopamine in the brain, with the former being used to treat anxiety and plants containing the latter being used as aphrodisics.

We do not know whether the medicine was intended to be ingested or used topically but based on the pharmacological literature it could have been used both ways. Of course, we must remember that not all traditional medicines were pharmacologically effective against the maladies they were intended to treat. medicinal ingredients would be combined in any ratio and in any quantity and administered in any way deemed appropriate without necessarily being medicinally effective. Traditional medicines are conceptualised within and resonate in harmony with cultural world views. Witchcraft and a belief in the supernatural realm were and remain prominent cultural influences in many Khoisan and Bantu-speaker societies. Traditional healers are specialised individuals who treat both physical and spiritual ailments.

Ironically, neither of the two plants that currently abound in the vicinity of the rock shelter, namely Helichrysum sp. and Senecio sp., both of which have medicinal and spiritual properties known to traditional medicine, are implicated in our GC-MS results (see references 82–84 for mass spectral information on these two species). Although speculative, it is perhaps worth noting that Helichrysum sp. has similar aromatic properties to Glycyrrhiza glabra and can be used as a substitute to foods. Given the faint liquorice odour it is possible that the medicine contained some Helichrysum. Although G. glabra is considered to be a colonial introduction to South Africa we should remember that plants thought to be recent, foreign introductions have been identified in far older archaeological deposits. Nor should we discount the possibility that there could be other plants in the vicinity that contain some of the same medicinal compounds present in G. glabra, but whose pharmacology is as yet undocumented.

While the paintings on the walls of La vie D’Antan are definitively San, we do not know whether they and the horn container are contemporary. That the horn container derived from domestic cattle does not necessarily suggest a khoi origin. Hunter-gatherers are known to have possessed cattle under certain circumstances and could easily have obtained the horn through barter, theft or scavenging. Both 19th-century khoi and San from the Western Cape shared a belief in a mythical animal known as the water bull, which bore a resemblance to domestic cattle and which was associated with, among other things, healing and whose horns were considered to have medicinal attributes. In the absence of archaeological deposits in the site, the medicine horn remains an isolated, chance discovery, but one which sheds new light on traditional medicines used in the Eastern Cape 500 years ago.

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Competing interests
We have no competing interests to declare.

Authors’ contributions
J.B.: Conceptualisation; writing – the initial draft; writing – revisions; project leadership; project management. S.W.: Methodology; sample analysis; data analysis. J.H.: Validation; writing – the initial draft. I.D.: Methodology; sample analysis; data analysis; validation; data curation; writing – the initial draft; writing – revisions.

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