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Molecular Characterisation of Bacterial Isolates from Vegetable, Cow Milk and Locust Bean Samples

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Authors' contributions

This work was carried out in collaboration among all authors. Author TAI designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors OOE and SOO managed the analyses of the study. Authors EBA and ORA and MAA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Molecular characterization involves characterization at molecular level without any effect of environment or development or physiological state of the organism. Biochemical characterization is the characterization of the biochemical state of the organism, which is in fact affected by environment, development as well as physiological state. The objective of this study was to molecularly characterize bacteria isolated from certain food samples. Five bacteria isolates were obtained from the vegetable samples while two LAB isolates were obtained from cow milk and locust bean samples. The bacteria isolates were identified using 16SRNA GENE sequencing using the BLAST algorithm and were identified as *Staphylococcus aureus CIP* 9973; *Pectobacterium carotovorum* subsp. *carotovorum* Pec 1; *Enterobacter cloacae* AS10 *Klebsiella aerogenes* OFM28; *Escherichia coli* 2013C-3342; *Proteus mirabilis* UPMSD3; *Lactobacillus plantarum* NCU116; *Lactobacillus plantarum* NRIC 0383. Characterization of bacteria isolates to molecular level is of enormous advantage as it helps to know the exact genus of a particular organism.

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1. INTRODUCTION

Modern technologies in food processing and microbiological food safety standards have reduced but not eliminated the likelihood of food related illnesses and product spoilage even in industrialized countries [1]. Food spoilage refers to the damage of the original nutritional value, texture, flavour of the food that eventually renders the food harmful and unsuitable to eat [2]. One of the major concerns in the food industry is contamination by pathogens, which are frequent causes of food borne diseases [3].

Consumption of vegetable products has dramatically increased in recent times. Vegetables are included among the basic and nutritious food for human beings. Vegetable, cow milk and locust bean samples are known veritable sources of important microorganisms [1]. Studies on microorganisms by [1 and 3, 4, 5] associated with vegetable, cow milk and locust bean samples followed the classical culture isolation technique and subsequent identification based on morphological, physiological and biochemical characteristics which depend largely on the cultivation of the microbes and thus a great variation (or even errors) may likely occur. These methods are also time-consuming and laborious [6]. Plate culturing techniques reveal only a little portion of the true microbial population in a natural ecosystem [8]. The increasing knowledge of gene sequences and the attendant development of new culture-independent molecular techniques are providing new and effective tools to analyze the diversity of microbial communities [9]. In this study, molecular methods were employed, to characterize and identify bacteria species associated with selected vegetables samples, cow milk and locust bean samples in order to reveal its true bacteria diversity.

2. METHODOLOGY

Fresh cow milk (500 ml) and locust bean (500 g) measured using a calibrated container and weighing scale (EMC LVD C-Tick Rohs, China) respectively were purchased from five different vendors at random locations in Modern Market, Benue state. All samples were kept at a temperature of 4^{o}C in an ice pack before being transported to the Microbiology Laboratory of the Federal University of Agriculture Makurdi for further analysis.

2.1 Processing of Samples

Vegetable samples got from the various markets were washed under running tap water. Twenty-five gram each of the vegetable samples (Tomato (*Solanum lycopersicum.*), Cucumber (*Cucumis sativus*),Cabbage (*Brassica oleracea*),Eggplant(*Solanum aethiopicum*), Green beans (*Phaseolus vulgaris*) and Fluted pumpkin leaves (*Telfairia occidentalis*) were weighed in sterile conditions, and reduced separately into smaller pieces using mortar and pestle, pre sterilized with 70% alcohol [10].

Locust bean sample (300 g) was weighed using a weighing scale, and crushed into tiny bits using a mortar and pestle pre sterilized using 70% alcohol. The different homogenates were collected in labeled sterile tubes and stored in sterile containers at 4ºC in the refrigerator (Haier Thermocool, China) for further use.

Raw cow milk was poured into a sterile stainless steel pot and heat slowly to 72 ^{o}C with stirring. The temperature was held at 72^{o}C for exactly 15 seconds after which the pot of milk was removed from the heat and placed in a large bowl filled with ice water [11]. Pasteurized raw cow milk sample was then transferred into sterile 500 ml beaker, covered with aluminum foil and left on the bench to ferment.

2.1.1 Isolation of bacteria from vegetable samples

Bacterial species were isolated from the vegetable samples using the serial dilution agar plate technique (12). One gram each of the processed vegetable products as described in sections 2.1 were aseptically transferred separately into 9 ml of sterile water to make a mixing stock. The homogenized mixture was serially diluted by taking 1 ml of stock mixture into appropriately labelled test tube to make 10^{-1} of the mixture. The serial dilution was continued until 10^{-7} and10^{-9} was obtained respectively for washed and unwashed vegetable samples. An aliquot (0.1 ml) of the different dilutions was then spread over nutrient agar media supplemented with amphotericin B (10 µg) before pouring to prevent fungal growth, the plates were then incubated for 24 hours at 37 ± 2^{o}C. Discrete colonies were then sub-cultured in nutrient broth and streaked over different selective and differential media agar plates i.e. MacConkey,

Eosin Methylene Blue and Mannitol salt agar. The media plates were properly labelled according to the vegetable samples and their source. Inocula in the various media plates were incubated in an incubator (Swiss *model*NU-5700, UK) at 37± 2^{o}C and observed for growth and colony formation after 24 hours.

2.1.2 Isolation of *Lactobacillus* species from cow milk and locust beans samples

Isolation of *Lactobacillus* species from cow milk and locust beans samples was done according to the method of (13). Processed locust beans and cow milk samples were serially diluted, by transferring 1 g/1 ml of each sample separately into 9 ml of sterile water to make a stock mixture. Homogenized mixture (1 ml) was then taken into appropriately labelled test tube to make 10^{-1} of the mixture. The serial dilution was continued until 10^{-8} was obtained. An aliquot (0.1 ml) of the respective dilutions was spread over MRS (de Man, Rogosa, Sharpe) agar plates. The MRS agar plates were then incubated anaerobically in an anaerobic jar (Microbiology AnaerotestX Merck, Darmstadt, Germany) at 37 ± 2^{o}C for 48 hours. Growth on MRS plates were then observed after 48 hours.

2.1.3 Purification of bacteria isolates

Colonies from cow milk and locust beans samples cultured on MRS agar plates were sub cultured twice on MRS agar and incubated in an incubator (Swiss *model* NU-5700, UK) at 37 ± 2^{o}C for 48 hours to get pure colonies. Purified isolates from vegetable, cow milk and locust bean samples were maintained at 4^{o}C (Haier Thermocool, China) in MRS and nutrient broth medium respectively for further characterization and identification.

2.1.4 Molecular identification of isolates from vegetable samples

2.1.4.1 Bacteria deoxyribonucleic acid extraction protocol

Fresh culture of isolates from vegetable samples were duplicated in sterile bottles containing nutrient broth for molecular identification. Broth culture (1000 µl) was centrifuged (Sorvall RC6 PLUS, Thermo-electron Corporation, Asheville, NC, USA) at 10, 0000 g for 5 min to get the pellets. The supernatant was decanted and 1ml of DNA Extraction Buffer (DEB) containing proteinase K (0.05 mg/ml) added and vortexed using a vortex mixer, after which 50 µl of 20% sodium dodecyl sulphate (SDS) was added and incubated in a water bath at 65 ^{o}C for 30 minutes. The tubes were then allowed to cool to room temperature, before adding 100 µl of 7.5 M potassium acetate and mixed briefly. The solution was then centrifuged (Sorvall RC6 PLUS, Thermo-electron Corporation, Asheville, NC, USA) at 13000 g for 10 minutes. The supernatant was transferred into fresh autoclaved tubes. Two third volumes of Isopropyl alcohol was then added to the supernatant in the test tubes and inverted gently before incubating in an incubator shaker (Thermo scientific) at 20 ^{o}C for 1 hour. After incubation the solution was centrifuged at 13000 g for 10 minutes and the supernatant discarded. Thereafter 500 µl of 70% ethanol was added and the supernatant further centrifuged again for 5 minutes at 13000 rpm. The supernatant was carefully discarded with the DNA pellet intact and the DNA pellets dried at 37 ^{o}C for 10-15 minutes. The pellets were later resuspended in 50 µl of Tris-EDTA (TE) buffer. Aliquots of DNA obtained were then stored at 20^{o}C in a refrigerator (Haier, thermocool China) for further laboratory analysis (14).

2.1.5 Polymerase Chain Reaction (PCR) analysis

Polymerase chain reaction was done according to the method of (15). Polymerase Chain Reaction sequencing preparation cocktail consisted of 10 µl of 5x Go Taq colourless reaction, 3 µl of 25 mM $MgCL_2$ (Magnesium Chloride), 1 µl of 10 mM of dNTPs (deoxyribonucleotide triphosphate) mix, 1 µl of 10 p mol each of the 16S rDNA gene forward primer (50-AGAGTTTGATCCTGGCTCAG-30),reverse primer: 50 (GTGTGACGGGCGGTGTGTAC-30), 0.3 units of Taq DNA polymerase (Promega, USA) made up to 42 µl with distilled water and 8 µl DNA template. Polymerase chain reaction was carried out in a Gene Amp 9700 PCR System Thermal cycler (Applied Bio system Inc., USA) with a PCR profile consisting of an initial denaturation at 94 °C for 5 min; followed by a 30 cycles consisting of 94°C for 30 seconds annealing of primer at 56 ^{o}C and 72 °C for 1 minute 30 seconds; and a final termination at 72 °C for 10 minutes. These was then allowed to chill at 4^{o}C in a refrigerator (Haier Thermocool, China).

2.1.6 Determination of the integrity of the deoxyribonucleic acid (DNA) Gel

Integrity of the DNA and PCR amplification product were checked on 1% agarose gel. Tris

Borate EDTA (1XTBE) buffer was used to prepare the agarose gel. The suspension was boiled in a microwave for 5 minutes. The molten agarose was allowed to cool to 60°C and stained with 3 µl of 0.5 g/ml ethidium. A comb was inserted into the slots of the casting tray and the molten agarose was poured into the tray. The gel was further allowed to solidify for 20 minutes to form the wells. Tris/Borate/EDTA (1XTBE) buffer was then poured into the gel tank to barely submerge the gel. Two microlitre (2µ l) of 10 x blue gel loading dye (which gives colour and density to the samples to make it easy to load into the wells and monitor the progress of the gel) was added to 4 µl of each PCR product and loaded into the wells after the 100 bp DNA ladder was loaded into well 1. The gel was electrophoresed at 120 V for 45 minutes and visualized with ultraviolet trans-illumination and photographed (14). The sizes of the PCR products were estimated by comparison with the mobility of a 100 bp ladder that ran alongside experimental samples in the gel.

2.1.7 Purification of amplified 16SrRNA gene product

Amplified fragments were further purified using 95% ethanol in order to remove the PCR reagents. A 3 M, 7.6 µl of Sodium acetate and 240 µl of 95% ethanol were added to each 40 µl PCR amplified product in a sterile 1.5 µl Eppendorf tube and mixed thoroughly using a vortex mixer (Cole-ParmerIndia, Pvt limited) and kept at -20°C in the refrigerator (Haier, Thermocool, China) for 30 minutes. This was then centrifuged (Sorvall RC6 PLUS, Thermo-electron Corporation, Asheville, NC, USA) for 10 minutes at 13000 rpm at 4°C followed by removal of the supernatant after which the pellet were washed by adding 150 µl of 70% ethanol and centrifuged for 15 min at 7500 g at 4°C in a refrigerator (Haier, Thermocool, China). All supernatant was repeatedly removed and tubes were inverted on paper tissue and left to dry in the fume hood at room temperature (28 ± 2^{o}C) for 10 -15 minutes after which it was suspended with 20 µl of sterile distilled water and kept at in the refrigerator (Haier, Thermocool, China) at -20 ^{o}C prior to sequencing. The purified gene product fragment was checked on a 1.5% Agarose gel ran on a voltage of 110 V for about 1 hour as previously described, to confirm the presence of the purified product and quantified using a Nano drop Model 2000 from thermo scientific UV-Vis spectrophotometers.

2.1.8 Sequencing of amplified fragments

Sequencing of amplified PCR fragments was carried out by Inquaba Biotec Sequencing Service, South Africa using the method of (16). Amplified fragments were sequenced using a Genetic analyzer 3130xl sequencer (Applied Bio systems) using the manufacturers' manual while the sequencing kit used was Big Dye terminator cycle sequencing kit. Bio- Edit software and MEGA 6 were also used for all genetic analysis. The obtained sequences were compared with those available in the Gen Bank database, using the Basic Local Alignment Search Tool (BLAST) at the National Center of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/).

2.1.9 Phylogenetic tree construction

A phylogenetic tree was constructed using the genetic distance neighborhood joining method of (16). MEGA 6.0 software was used for the construction.

3. RESULTS

A total of five [5] bacteria were isolated from washed tomatoes, eggplant and fluted pumpkin samples, while the least number of three [3] bacteria (*Staphylococcus aureus, Bacillus* and *Pseudomonas* species) were isolated from cabbage sample. The bacteria isolates were labelled numerically before molecular identification (Table 1).

Bacteria isolates from locust beans and fermented cow milk samples were identified as A B and C prior to molecular analysis. Isolates A and B were gotten from locust beans samples, while isolate C in addition to A and B were from fermented cow milk sample Occurrence of bacteria isolates from locust beans and fermented cow milk samples are outlined in Table 2.

Sharp band amplicons corresponding to the 16S rRNA intergenic spacer region was observed for the test bacteria and bacteriocin producing LAB. The negative control without any template did not give any band (Plate 1). Genetic distance between all isolates is depicted in Fig. 1. Similarity searches with sequences in the ribosomal data project database revealed 82.30% identity with *Staphylococcus aureus*CIP 9973, 94.10% with *Pectobacterium carotovorum* Pec1, 97.70% with *Enterobacter cloacae* AS10,

93.80% with *Klebsiella aerogenes* OFM28, 91.80% with *Proteus mirabilis* UPMSD3, 94.50% with *Escherichia coli* 2013C-3342, 95.90% identity with *L. plantarum* NRIC 0383 and 83.40% with *L. plantarum*NCU116 (Table 3).

Phylogenetic tree constructed using the neighbor joining method showed that the isolates were divided into three clusters (Groups). Group three (3) was further divided into subgroups. The first group consisted of *Lactobacillus plantarum* NCU116 and *Lactobacillus plantarum* NRIC 0383, while the second group consisted of *Staphylococcus aureus* CIP 9973 alone, the third consisted of *Escherichia coli* 2013C-3342, *Enterobacter cloacae* AS10, *Klebsiella aerogenes* OFM28, *Proteus mirabilis* UPMSD3 and *Pectobacterium carotovorum* Pec1 (Fig. 2).

4. DISCUSSION

Bacteria isolated from selected vegetables used in this study belonged to eight genera identified as *Enterobacter aerogenes, Staphylococcus aureus, Escherichia coli, Klebsiella*, *Proteus, Pseudomonas, Pectobacterium* and *Bacillus* species. With the exception of *Pectobacterium carotovorum* all the bacteria identified in this study were also isolated and identified by [5];

Table 1. Bacteria occurrence in washed vegetable samples

Vegetable samples	Bacterial Isolates Codes
Tomatoes (*Solanumlycopersicum*)	1, 2, 3, 4 and 5
Cucumber (*Cucumi ssativus*)	1, 6, 3 and 5
Cabbage (*Brassica oleracea*)	1, 5 and 7
Eggplant (*Solanum aethiopicum*)	1, 2, 4, 7 5.
GreenBeans (*Phaseolus vulgaris*)	2, 4, 7 and 6
Pumpkin (*Telfairia occidentalis*)	2, 3, 5, 1 and 7

Table 2. Occurrence of *Lactobacillus* Isolates in locust beans and cow milk samples

Point of collection	Sample	*Lactobacillus* occurrence
Vendor A	Locust bean	A
Vendor B	Locust bean	A, B
Vendor C	Locust bean	A B
Vendor A	Fermented milk	A B C
Vendor B	Fermented milk	A
Vendor C	Fermented milk	B

	8	7	1	5	6	2	4	3
8		0.15	0.93	0.91	0.85	0.87	0.89	0.86
7	0.15		1.00	0.98	0.92	0.93	0.96	0.92
1	0.93	1.00		0.35	0.29	0.31	0.33	0.30
5	0.91	0.98	0.35		0.13	0.16	0.21	0.18
6	0.85	0.92	0.29	0.13		0.10	0.15	0.12
2	0.87	0.93	0.31	0.16	0.10		0.17	0.14
4	0.89	0.96	0.33	0.21	0.15	0.17		0.15
3	0.86	0.92	0.30	0.18	0.12	0.14	0.15	

Fig. 1. Genetic distance between molecularly characterized isolate

1: Staphylococcus aureus CIP 9973; 2: Pectobacterium carotovorum subsp. carotovorum Pec 1; 3: Enterobacter cloacae AS10 4: Klebsiella aerogenes OFM28; 5: Escherichia coli 2013C-3342; 6: Proteus mirabilis UPMSD3; 7(A): Lactobacillus plantarum NCU116; 8(B): Lactobacillus plantarum NRIC 0383

Table 3. Similarity searches of bacteria isolates with sequences in the ribosomal project database

Isolate codes	Pairwise identity (%)	NCBI accession	Organism description
1	82.30%	MG650162	*Staphylococcus aureus* partial 16S rRNA geneCIP 9973
2	94.10%	MH532568	*Pectobacterium carotovorum* subsp. *carotovorum* strain Pec1 16S ribosomal RNA gene, partial sequence
3	97.70%	MH605571	*Enterobacter cloacae* strain AS10 16S ribosomal RNA gene, partial sequence
4	93.80%	MH542333	*Klebsiella aerogenes* strain OFM28 16S ribosomal RNA gene, partial sequence
5	91.80%	MH393635	*Proteus mirabilis* strain UPMSD3 16S ribosomal RNA gene, partial sequence
6	94.50%	CP027766	*Escherichia coli* strain 2013C-3342 chromosome, complete genome
A	83.40%	CP016071	*Lactobacillus plantarum* strain NCU116, complete genome
B	95.90%	AB362652	*Lactobacillus plantarum* gene for 16S rRNA, partial sequence, strain NRIC 0383

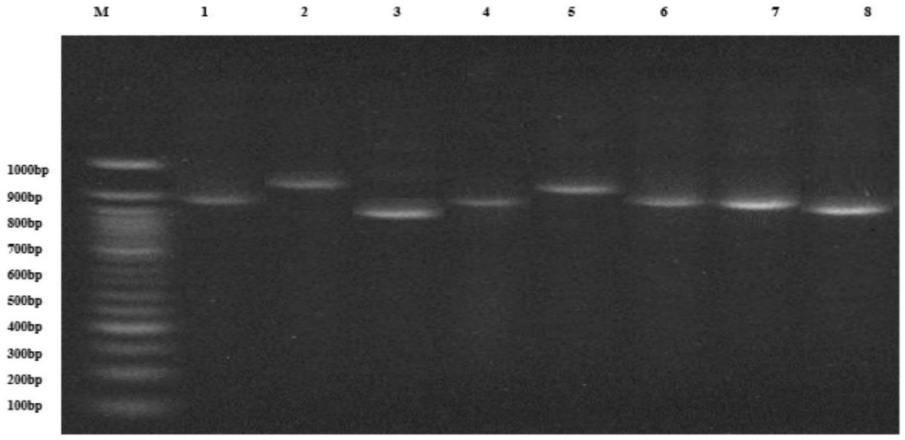

Plate 1. Polymerase chain reaction amplification product of bacteria isolates from visualized by agarose gel electrophoresis

Lane M: 100bp marker; Lane 1: Staphylococcus aureus CIP 9973; Lane 2: Pectobacterium carotovorum subsp. carotovorum Pec1; Lane 3: Enterobacter cloacae AS10; Lane 4: Klebsiella aerogenes OFM28; Lane 5: Escherichia coli 2013C-3342; Lane 6: Proteus mirabilis UPMSD3; Lane 7 (A): Lactobacillus plantarum NCU116; Lane 8 (B) Lactobacillus plantarum NRIC 0383

[17]; [18] and [19] from vegetable samples in different countries and climates. Some of the bacteria isolated in this study may be part of the natural flora of the vegetables or contaminants from soil, irrigation water and the environment. Other sources of contamination could be the transportation means, washing or rinsing water or handling by processors [18].

The presence of lactic acid bacteria (LAB) in cow milk and locust been used in this study was an evidence of LAB as a normal microflora of fermented foods [8; 20]. It also shows that traditional fermented food products can potentially be good sources of probiotic organisms [21] producing antimicrobial substances such as organic acids, diacetyl, hydrogen peroxide and bacteriocins which are suspected to be associated with the preservation of many fermented food condiments in Nigeria [22]. [23] also reported diverse groups of microorganisms including *Bacillus, Micrococcus, Leuconostoc, Staphylococcus, Enterobacteriaceae* and lactic acid bacteria (LAB) that play active roles in the process of fermentation. different types of carbohydrates.

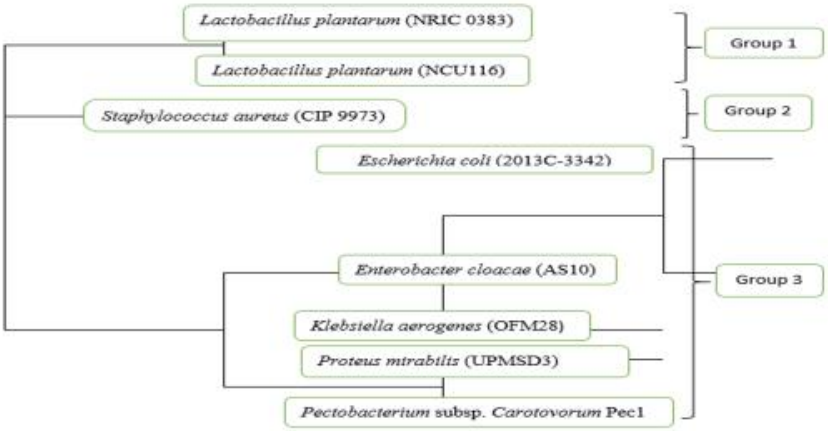

Fig. 2. Phylogenetic tree showing position of bacteria inferred by the neighbor-joining method

Genetic distance between loci as determined by the frequency with which recombination events occurred between the genes was useful as it showed the degree of genetic difference between the species. Homology searches of 16s rDNA sequences of the bacteria isolates which showed 82.30% identity with *Staphylococcus aureus* CIP 9973, 94.10% with *Pectobacterium carotovorum* Pec1, 97.70% with *Enterobacter cloacae* AS10, 93.80% with *Klebsiella aerogenes* OFM28, 91.80% with *Proteus mirabilis* UPMSD3, 94.50% with *Escherichia coli* 2013C-3342, 95.90% identity with *L. plantarum* NRIC 0383 and 83.40% with *L. plantarum*NCU116 using the BLAST algorithm indicated the extent to which two (nucleotide or amino acid) sequences have the same residues at the same positions in an alignment. These results showed the importance of using molecular methods for identifying newly isolated microorganisms. According to [22] genotypic methods are independent of variations in growth conditions, if species-specific primers or probes are available; these offer a very fast method of confirming the identity of the target organism. Furthermore, identification of LAB based on the morphological, physiological and biochemical characteristics are often considered as unreliable, since different species may have similar morphological and nutritional requirements. Phenotypic characterization based on sugar fermentation profile may be used as a presumptive identification but does not provide reliable identification [23]. However genotype-based methods such as 16S rDNA are robust to identify bacteria as a complement or alternative to phenotypic methods [22].

5. CONCLUSIONS

Molecular characterization can play a pivotal role in uncovering the history, and estimating the diversity, distinctiveness and population structure. Genetic distance between loci as determined by the frequency with which recombination events occurred between the genes was useful as it showed the degree of genetic difference between the species. Characterization of bacteria isolates to molecular level is of enormous advantage as it helps to know the exact genus of a particular organism. Awareness of the level of genetic diversity and the proper management of genetic resources are important issues in modern scenario.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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