



Effect of Abiotic Factors on the Antifungal Activity of *Lactobacillus* Strains Isolated from Commercial Dairy and Fermented Foods from Federal Capital Territory, Nigeria

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Authors' contributions

This work was done in collaboration among all the four authors. Author MA designed the study and supervised the work. Author II wrote the first draft of the manuscript and performed the analysis. Authors NA and PO supervised the study and analyzed the data. All the authors managed the literature search writing of the final manuscript, read and approved the final manuscript.

Article Information

DOI: 10.9734/EJNFS/2021/v13i130350

Editor(s):

(1) Dr. Dan-Cristian Vodnar, University of Agricultural Science and Veterinary Medicine, Romania.

Reviewers:

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(2) Amit Ranjan, Tamil Nadu Dr. J. Jayalalithaa Fisheries University, India.

Complete Peer Review History: <http://www.sdiarticle4.com/review-history/64257>

Original Research Article

Received 25 October 2020
Accepted 29 December 2020
Published 02 March 2021

ABSTRACT

Increasing consumer demand for natural products have renewed food industry attention in bio preservation. Lactic acid bacteria are of particular interest as effective alternative to chemical preservation because of their food grade status. This work explores the effect of antifungal compounds produced by isolates of *Lactobacillus* sp on some selected pathogenic fungi growth. Samples of dairy and fermented products were purchased from commercial vendors within the Federal Capital Territory (FCT) and screened for the presence of *Lactobacillus* sp. The *Lactobacillus* sp isolated were screened for antifungal activity against *Aspergillus fumigatus*, *Candida albicans* and *Trichophyton rubrum* using a dual culture assay. Strains with antifungal activity were identified and the fungal inhibitory activity was further evaluated. The effect of abiotic factors on the antifungal activity was evaluated by overlay assay under different temperature and

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pH. Majority of the identified isolates belonged to the genus *Lactobacillus*. *Lactobacillus* sp. produced antifungal compounds under different temperatures (25°C, 30°C and 37°C). The antifungal compounds produced by *Lactobacillus* strains showed greater inhibitory activity on *Aspergillus fumigatus*. At 30°C the percentage zones of inhibition range were 44.4%- 60.4%. All isolates showed stronger antifungal activity when grown at pH 4.0 and 5.0. At a pH 2.0 there was a total inhibition of fungal growth however, there was no inhibition of fungal growth at the pH 7.0. Lactic acid bacteria can be employed as effective alternative to chemical preservatives in food. Temperature and pH of the culture medium could influence the production of antifungal compounds by lactic acid bacteria.

Keywords: *Lactobacillus*; antifungal; overlay assay; abiotic factors; fermented foods; lactic acid bacteria.

ABBREVIATIONS

LAB : Lactic Acid Bacteria
MRS : de Man's Rogosa and Sharpe
AMAC : Abuja Municipal Area Council

1. INTRODUCTION

Increasing consumer demand for natural product has renewed the attention of the food industry to bio-preservation. Lactic acid bacteria are of particular interest as effective alternative to chemical preservation because of their food grade status [1,2]. Some LAB have the ability to produce inhibitory growth compound, in the final fermentation products [3], these compounds are known: formic, propionic, butyric, valeric, caproic, phenyllactic and reuterin [4]; acetic and lactic acid [5] and has the ability to retard or eliminate mycelia growth and spore germination, either on their own or synergistically [6].

The future perspective is to use these antifungal lactic acid bacteria in food or feed products to decrease the growth of deleterious fungi. Moulds and yeasts are significant spoilage organisms of food and feed. In addition, the potential production of toxic and carcinogenic mycotoxins by moulds is of particular concern. Besides this problem, fungal spoilage of food also causes significant economic losses. Worldwide, about 5-10% of the food production is estimated to be spoiled by these organisms [7].

Bio preservation refers to the use of a microflora, natural or controlled, or its antibacterial products to extend shelf-life and enhance the safety of foods [8]. The primary preservation effect achieved by LAB is due to the production of lactic acid, which lowers the pH and also directly inhibits many microorganisms [9]. Besides the production of lactic acid, LAB produces other antimicrobial substances, such as acetic acid, hydrogen peroxide, diacetyl, reuterin and

bacteriocins, which might play an important role in their preserving capabilities [10].

The idea that LAB prevents intestinal disorders and diseases is nearly as old as the science of microbiology [11]. Therefore, in the development of probiotic food intended for human consumption, strains of LAB have most commonly been used. The term "probiotic" (Greek: for life) was improved and documented by Fuller, 1989 [12] with the following definition: "A live microbial food supplement which beneficially affects the host animal by improving its intestinal microbial balance".

Aspergillus sp. and *Penicillium* sp. and yeasts are most frequently found in food compared to most filamentous fungi due to their fastidious nature. Many foodstuffs are rich substrates containing necessary carbohydrates, amino acids and essential vitamins and minerals, making them ideal growth substrates for many yeast species.

The objectives of this study was to isolate lactic acid bacteria with antifungal properties and to find the factor that increases the production of antifungal compounds by *Lactobacillus* sp.

2. MATERIALS AND METHODS

2.1 Media

Nutrient agar (Oxoid, UK) , de Man's Rogosa and Sharpe (MRS) agar (Oxoid, UK), peptone water (Oxoid, UK), Sabouraud dextrose agar (Oxoid, UK) and malt extract agar (Oxoid, UK).

2.2 Microorganisms

The fungal strains used were *Trichophyton rubrum*, *Aspergillus fumigatus* and *Candida albicans* where obtained from the Department of Microbiology and Biotechnology, National

Institute for Pharmaceutical Research and Development, Abuja.

2.3 Sample Collection

The study was conducted between Jan - June, 2014. Fresh samples of fermented local foods- Nunu (cow milk), Kunun-zaki (millet) and Ogi (maize) were purchased from commercial vendors from Four Area Councils (AMAC, Kuje, Bwari, Gwagwalada) within the Federal Capital territory (FCT), Nigeria and transported immediately in an ice box to the laboratory within 3 hours of collection. Ten grams of the samples were suspended into 90 mls of peptone water and spread on MRS agar then incubated at 37°C for 24 hours. The colonies isolated were further purified by streaking on MRS agar and maintained on nutrient agar slant at 4°C.

2.4 Preliminary Screening and Isolation of *Lactobacillus* sp

The isolated colonies were subjected to morphological, cultural, physiological and biochemical techniques according to Bergey's manual [13]. The morphological parameters employed include Gram staining, endospore staining and cell morphology test; biochemical tests- indole, catalase, sugar fermentation, oxidase, motility, triple sugar iron reaction, starch hydrolysis, urease. The isolates confirmed to be *Lactobacillus* sp were preserved in MRS broth supplemented with glycerol and stored at -20°C.

2.5 Antagonistic Activities of *Lactobacillus* Isolates against Fungal Pathogens at Different Temperatures

This was done by adopting the agar overlay method as described by Magnusson et al., 2003 [14]. Simply a suspension of the overnight culture of *Lactobacillus* sp was suspended in normal saline and the inoculum adjusted to match 0.5 McFarland standard (10^8 cfu/ml). A 100 fold serial dilution was done in MRS broth to achieve a concentration of 10^6 cfu/ml. The standardized inoculum was then streaked as 2-cm wide lines on MRS agar and incubated at 25 °C, 30 and 37 °C for 48 h. After incubation, 10ml of soft malt extract (0.7%) agar seeded with 0.1ml of standardized fungi (1.0×10^5 cfu/ml) determined by counting on a Neubauer counter haemocytometer were then poured onto the agar plates. The plates were incubated at 30°C for 72

h. The screening was repeated at incubation temperatures of 30 and 37°C. The diameter zones of inhibition were recorded and the percentage of inhibition calculated as the area of inhibited growth in relation to the total area of the petri dish.

2.6 Antifungal Activities of *Lactobacillus* Isolates at Different pH

A single colony was sub-cultured in normal saline and adjusted to match 0.5 Mac Farland standard (10^8 cfu/ml). Thereafter, 100 fold serial dilution was done in MRS broth to achieve concentration 10^6 cfu/ml. The standardized inoculum was then streaked as two lines on MRS agar with pH range (2, 3, 4, 5 and 7) and incubated at 30°C for 48 h. Hydrochloric acid (0.1 M) and Sodium hydroxide (0.1 M) were used to achieve the required pH. After incubation 10ml of soft malt extract (0.7%) agar seeded with 0.1ml of standardized fungi (1.0×10^5 cfu/ml) were then poured onto the agar plates. The plates were incubated at 30°C for 72 h. The percentage of inhibition were calculated as the area of inhibited growth in relation to the total area of the petri dish [14].

3. RESULTS

3.1 Isolation

A total of 50 lactic acid bacteria were isolated from the samples, identified as Gram positive rod shaped bacilli, catalase negative, non-spore forming, non-motile and round, creamy white colonies on MRS agar (Table 1). The isolates demonstrated the best growth at temperature 30 - 37°C, optimum pH for all of the isolates was 5.0 - 6.5. The biochemical characteristics of the isolates are shown in Table 2. A total of 19 isolates were confirmed to be *Lactobacillus* sp, out of which only 16 possessed antifungal activity while 3 (LN3, LN4 and LN8) did not have antifungal potential.

3.2 Effect of Temperature on the Antifungal Activities of *Lactobacillus* sp Against *Aspergillus Fumigatus*

Out of 19 isolates confirmed to be *Lactobacillus* sp only 10 showed antifungal activity against *Aspergillus fumigatus* (Table 3). The strongest antifungal activity (30.8% - 60.4%) was displayed at 30°C.

3.3 Effect of Temperature on the Antifungal Activities of *Lactobacillus* sp Against *Trichophyton rubrum*

Out of 19 isolates confirmed to be *Lactobacillus* sp only 9 showed antifungal activity against *Trichophyton rubrum* (Table 4). The percentage zones of inhibition range between 1.2 - 25%. The results showed stronger inhibition at 30°C.

3.4 Effect of pH on the Antifungal Activity of *Lactobacillus* sp

A total inhibition of the growth of *Aspergillus fumigatus* and *Trichophyton rubrum* by the *Lactobacillus* sp was observed at the pH 2.0, but the use of malt extract agar with pH at 3.0, 4.0 and 5.0 showed a decrease in the antifungal activity of the *Lactobacillus* sp. The isolates lost their antifungal activity at pH 7.0 showing no sign of inhibition to the fungi (Figs. 4 and 5).

Table 1. Origin of isolates from fermented food and dairy product after screening for *Lactobacillus*

Source	No. of isolates (LAB)	No. of <i>Lactobacillus</i> sp	<i>Lactobacillus</i> Isolates
Pap (Maize)	15	5	LP1, LP2, LP3, LP4, LP5
Kunu (Millet)	17	6	LK1, LK2, LK3, LK4, LK5, LK6
Nunu (Cow milk)	18	8	LN1, LN2, LN3, LN4, LN5, LN6, LN7, LN8
Total	50	19	

Key LAB- lactic acid bacteria

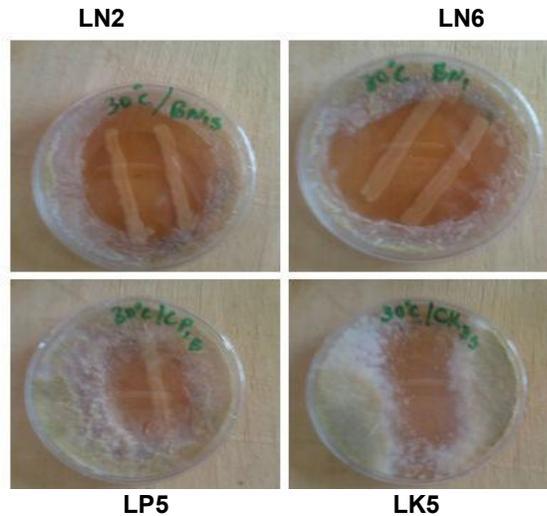


Fig. 1. Antifungal activity of LAB against *A. fumigatus* at temperature 30 °C

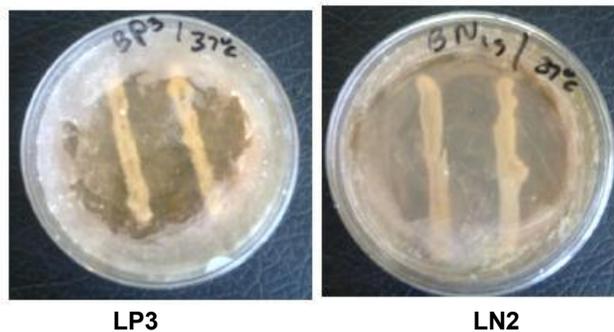


Fig. 2. Antifungal activity of LAB against *T. rubrum* at temperature 30 °C

Table 2. Phenotypic characterization of *Lactobacilli* isolates

Morphological Characteristics	<i>L. fermentum</i>	<i>L. plantarum</i>	<i>L. delbrueckii</i>	<i>L. casei</i>	<i>L. lactis</i>
No of isolates	4	6	4	3	2
Colour	creamy white	creamy white	creamy white	creamy white	creamy white
Shape	Rods	Rods	rods	Rod	Short Rods
Look on media	Smooth round colonies	Smooth round colonies	smooth round colonies	smooth round colonies	smooth round colonies
Gram stain	+	+	+	+	+
Spore forming	-	-	-	-	-
Motility	non motile	non motile	non motile	non motile	non motile
pH optimum	5.0-6.5	5.0-6.5	5.0-6.5	5.0-6.5	5.0-6.5
Temperature	30° -37 °C	30° -37 °C	30°-37 °C	30°-37 °C	30° -37 °C
Catalase activity	-	-	-	-	-
H2S formation	-	-	-	-	-
Urease	-	-	-	-	-
Co2 from glucose	acid and gas	Acid	acid	Acid and gas	Acid
Starch hydrolysis					
T S I reaction	acid and gas	Acid	acid	Acid and gas	Acid
Carbon fermentation					
D-Glucose	+	+	+	+	+
Galactose	+	+	-	+	-
D-Fructose	+	-	+	+	+
Surcose	+	+	+	-	+
Maltose	+	+	+	+	+
Mannitol	-	+	-	+	-
Sorbitol	-	+	-	-	-
Lactose	-	+	-	-	+
Mannose	+	+	+	+	+

Table 3. Antifungal activities of *Lactobacillus* sp against *Aspergillus fumigates*

LAB Strains	Zone of inhibition (mm)			Percentage inhibition (%)		
	25°C	30°C	37°C	25°C	30°C	37°C
LP3	60.0	50.0	30.0	44.4	30.8	11.1
LN2	50.0	70.0	50.0	30.8	60.4	30.8
LP1	40.0	60.0	50.0	19.7	44.4	30.8
LN5	50.0	65.0	60.5	30.8	52.1	52.1
LK5	60.0	35.0	40.0	44.4	15.1	19.7
LP5	40.0	40.0	30.0	19.7	19.7	11.1
LP2	20.0	30.0	10.0	4.9	11.1	1.2
LK6	20.0	55.0	40.0	4.9	37.3	19.7
LK1	10	25.0	20.0	1.2	7.7	4.9
LN6	30.0	40.0	30.0	11.1	19.1	11.1

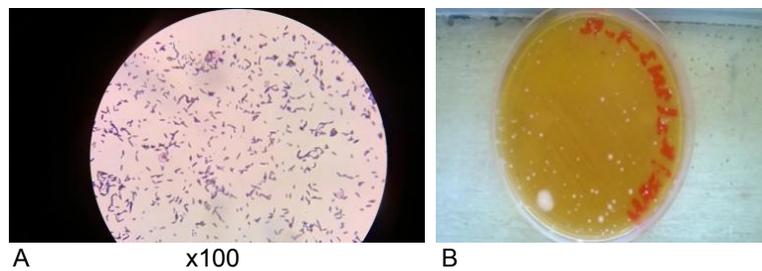


Fig. 3. Isolated *Lactobacillus* strain by Gram staining (A) and on MRS agar (B)

Table 4. Antifungal activities of *Lactobacillus* sp against *Trichophyton rubrum*

LAB Strains	Zone of inhibition (mm)			Percentage inhibition (%)		
	25°C	30°C	37°C	25°C	30°C	37°C
LP4	40.0	45.0	30.0	19.7	25	11.1
LK3	40.0	35.0	40.0	19.7	15.1	19.7
LN7	20.0	40.0	30.0	4.9	19.7	11.1
LK4	30.0	25.0	20.0	11.1	7.7	4.9
LN6	20.0	20.0	30.0	4.9	4.9	11.1
LK2	30.0	30.0	20.0	15.1	11.1	4.9
LP1	20.0	20.0	10.0	4.9	4.9	1.2
LN1	15.0	20.0	10.0	2.8	4.9	1.2
LN2	10	10.0	10.0	1.2	1.2	1.2

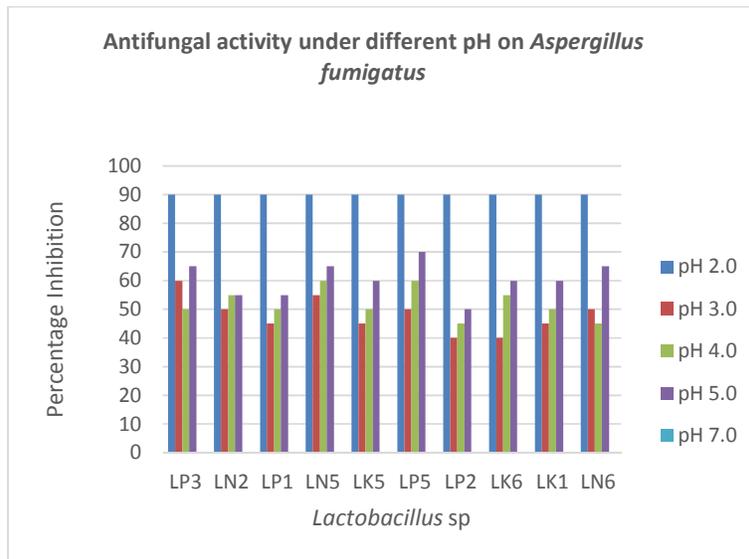


Fig. 4. Antifungal activity under different pH on *Aspergillus fumigates*

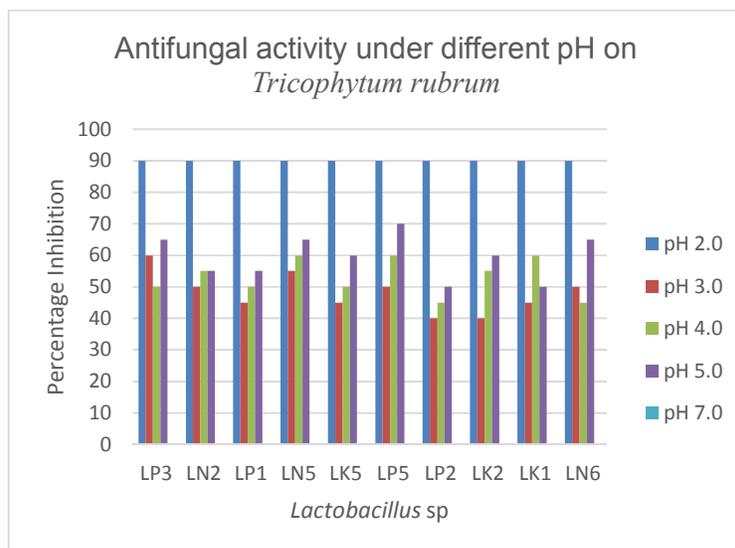


Fig. 5. Antifungal activity under different pH on *Trichophyton rubrum*

4. DISCUSSION

4.1 Isolation

Lactobacillus strains play an important role in food fermentation processes. Modern concepts or perspectives of the application of *Lactobacillus* strains include the following selections; the best adapted and safe for human application as it is an important bio-defence factor in human intestinal tract, non-pathogenic, with probiotic effects and/or health-promoting effects and food protective activities. Results from this study indicate that *Lactobacillus* sp are good potential candidate for probiotic product preparation or as food additives as well as antimicrobial activity against human pathogens and antifungal activity against food spoilage organisms.

None of the *Lactobacillus* isolates possessed inhibitory activity against *Candida albicans*. This is contrary to a study by Collins and Hardt, 1980 [15] which reported filtrate of a culture of *Lactobacillus acidophilus* isolated from the intestinal tracts showed slight inhibition in the growth of *Candida albicans*. Rossoni et al., 2018 [16], reported the antifungal activity of clinical *Lactobacillus* strains against *C. albicans* biofilms. The inactivity of the LAB strains may be linked to their source and geographical location.

4.2 Effect of Temperature on the Antifungal Activities of *Lactobacillus* sp against *Aspergillus fumigatus*

The strongest antifungal activity (30.8% - 60.4%) was displayed at 30°C. This agrees with previous studies by Rouse et al. [17] and Laref et al. [18] where they reported 30°C as the optimum and ideal temperature for the production of antifungal compounds by *Lactobacillus plantarum* on MRS agar. The highest antifungal inhibition against *Aspergillus fumigatus* was gotten from LAB isolated from locally produced dairy product (nunu) with a percentage inhibition of 60.0% at 30°C. The fermented foods (pap and kunu) gotten from cereals produced lower inhibition of 44% at 25°C and 30°C.

The large zone of inhibition against *Aspergillus fumigatus* was observed by LN2, LN1 and LK3 at 30°C, the percentage of inhibition by these strains was 52.1, 60.4 and 44.4% (Table 3). Generally, it was observed that incubation at 30°C produced the best antifungal activity against *A. fumigatus* (Table 3). This result agrees

with a study by Amal and Saadyh, [19], that reported the antifungal potential of *L. plantarum* from yoghurt against *A. fumigatus* at 30°C. *A. fumigatus* is a known contaminant in dairy foods and its inhibition by LAB as a probiotic is of great importance [19]. Similar temperatures (30°C) were suggested by Rouse et al., [17] and Binachini, [20] as ideal for the production of antifungal compounds by *L. plantarum* in MRS broth. Maganusson and Schnurer, [4] also reported incubation at 30°C as ideal for maximum production of antifungal compounds by *Lactobacillus coryniformis* in MRS broth.

4.3 Effect of Temperature on the Antifungal Activities of *Lactobacillus* sp against *Trichophyton Rubrum*

The LAB isolates showed lower antifungal activity against *Trichophyton rubrum* with the highest percentage of inhibition 25% at 30°C (Table 4). Roy et al. [21] studying the production of antifungal compound by *Lactococcus lactis* reported optimum incubation temperature at 30°C.

4.4 Effect of pH on the Antifungal Activity of *Lactobacillus* sp

At lower pH range 2-4 the *Lactobacillus* isolates produced stronger antifungal activities with 90% inhibition when grown at pH 2 however as the pH increased there was a reduction in antifungal activity with a total loss of activity at pH 7 (Figs. 4 and 5). This agrees with a study by Rouse et al., [17] which reported that *L. plantarum* produced the antifungal compounds in the lower pH range. However, Maganusson and Schnurer, [4] reported that their strain of *L. coryniformis* produces the antifungal compounds in the highest pH 6.5. *Lactobacillus plantarum* isolated by Bianchini, [20] showed more inhibition of mold growth towards the higher pH levels tested with pH 5-7. Falguni et al. [22] suggested that the maximum production of antifungal compounds by *L. brevis* NCDC at pH 6 and 7 in MRS broth.

The temperature and pH of the culture medium influence the production of the antifungal compounds by lactic acid bacteria. These factors affect the metabolism of the bacteria, therefore altering the production of antifungal compounds. There was total inhibition of the growth of *Aspergillus fumigatus* and *Trichophyton rubrum* at the pH 2.0 (Figs. 4 and 5) which was most probably due to the secretion of organic acids

which depends strongly on the pH, since they are active in the dissociated form. In this form, their lipophilic condition permits them to penetrate across the membrane. At a higher intracellular pH, the acid dissociates to release protons and conjugate bases, which disrupt the membrane proton motive force [23], since lactic acid bacteria produce organic acids. These might also activate other antifungal compounds such as peptides by lowering the pH, these compounds are therefore eliminated by neutralization [4]. The antifungal compounds produced have potential to be used as food bio preservation to inhibit conidia germination and mycelia growth of spoilage and pathogenic fungi depending on food type, and pH of food especially in heat, and cold processed foods.

5. CONCLUSION

This study shows that *Lactobacillus* sp from dairy and locally fermented products were found to have an antifungal activity against *Aspergillus fumigatus* and *Trichophyton rubrum* which was influenced by temperature and pH of the culture medium.

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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The peer review history for this paper can be accessed here:
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