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Assessing probiotic attributes and molecular characterization of *Weissella confusa* from barbari goat faeces using 16S rRNA

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Abstract

The present study examined the probiotic potential and molecular characterization of *Weissella confusa* isolated from goat faeces using *16S rRNA* gene sequencing. A total of 15 lactic acid bacterial isolates were obtained from 42 fresh faecal samples collected from healthy barbari goats. Based on morphological, biochemical, and molecular analyses, two isolates goat faecal-7 (GF-7) and goat faecal-8 (GF-8) (Genbank accession numbers: MZ314055.1 *Weissella confusa* India and MZ314055.2 *Weissella confusa* India) were identified as *W. confusa* with 99.2% similarity to reference sequences in GenBank. The isolates demonstrated significant probiotic attributes including acid tolerance (survival at pH 2.5), bile salt resistance (0.3% oxgall), adhesion to intestinal epithelial cells (34.2%), and antimicrobial activity against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923. Antibiotic susceptibility testing revealed sensitivity to ampicillin, chloramphenicol, and tetracycline, indicating their safety for potential probiotic applications. The strains produced lactic acid at a concentration of 8.4 g/L and exhibited β-galactosidase activity. Phylogenetic analysis confirmed that the isolates belong to the Weissella genus. The results indicate that *W. confusa* MZ314055.1 and MZ314055.2 possess probiotic characteristics suitable for functional food development and animal health enhancement.

1. Introduction

Probiotics are defined as live microorganisms that, when administered in sufficient quantities, impart health benefits to the host organism (Hill et al. 2014). In recent years, the rising demand for natural and functional foods has stimulated extensive research into probiotic microorganisms, with particular emphasis on lactic acid bacteria (LAB), which are widely regarded as safe for consumption and classified as Generally Recognized as Safe (GRAS) (Salvetti et al. 2012). Within this group, the genus *Weissella* has attracted increasing scientific interest due to its emerging probiotic potential as well as its technological relevance in food fermentation processes. Species of *Weissella* are obligately heterofermentative LAB that metabolize glucose into lactic acid, carbon dioxide, and ethanol or acetic acid (Fusco et al. 2015). These bacteria are distributed across a broad range of ecological habitats, including fermented food products, plant-associated environments, and the gastrointestinal tracts of animals. Among them, *Weissella confusa* has been reported from diverse sources such as traditional fermented foods, fresh vegetables, and the intestinal microbiota of animals (Chen et al., 2014). This ecological versatility underscores its adaptability and highlights its potential as a candidate for probiotic exploitation and functional food applications.

Accurate identification and characterization of probiotic bacteria require both phenotypic and molecular methods. Among these, 16S ribosomal RNA (rRNA) gene sequencing is considered the gold standard for bacterial identification and phylogenetic analysis because of its conserved and variable regions, which enable species-level discrimination (Janda and Abbott 2007). This molecular method supports reliable taxonomic identification and facilitates the construction of phylogenetic relationships among bacterial species. The assessment of probiotic potential involves evaluating various functional properties including acid and bile tolerance, adhesion to intestinal epithelial cells, antimicrobial activity against pathogens, and safety parameters such as antibiotic susceptibility (FAO/WHO 2006). These characteristics are essential for the survival and functionality of probiotic bacteria in the hostile environment of the gastrointestinal tract. Despite recent studies demonstrating the probiotic potential of *Weissella* species, comprehensive characterization of *Weissella confusa* isolated from goat faecal sources is limited. The objectives of this study are to isolate and identify *W. confusa* from goat faeces using *16S rRNA* gene sequencing, evaluate probiotic attributes such as acid tolerance, bile resistance, adhesion properties, and antimicrobial activity, and assess the safety profile through antibiotic susceptibility testing.

2. Materials and Methods

2.1 Ethical approval

All the experimental procedures were conducted with prior approval from the Institutional Animal Ethical Committee (IAEC), Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut-250110 (Registration No. 250/GO/Re/SL/19/CPCSEA) in compliance with the regulations of Committee for Control Supervision of Experiments on Animals (CCSE), Ministry of Fisheries, Animal Husbandry and Dairying, Government of India.

2.2 Sample collection and bacterial isolation

Fresh faecal samples were collected from healthy Barberi goats (n = 42) maintained at the Livestock Farm Complex, Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut in sterile containers and transported to the laboratory within 2 hours. One gram of each sample was homogenized in 9 mL of sterile phosphate-buffered saline (PBS) and serially diluted. The 4^{th}, 5^{th} and 6^{th} dilutions were plated on de Man, Rogosa, and Sharpe (MRS) agar (pH 6.2) and incubated at 37°C for 48 hours under anaerobic conditions.

2.3 Morphological and biochemical characterization

Isolated colonies were examined for morphological characteristics including colony appearance, cell morphology using Gram staining, as well as catalase activity. Biochemical profiling was conducted by carbohydrate fermentation tests using sugars *viz*. cellobiose, galactose, lactose, maltose, mannitol, mannose, and sucrose (Himedia, India).

2.4 Molecular identification and phylogenetic analysis

DNA extraction was performed using the Thermo Scientific GeneJET Genomic DNA Purification Kit (#K0721) according to the manufacturer's instructions. The 16S rRNA gene was amplified using universal primers 784F (5′-AGGATTAGATACCCTGGTA-3′) and 1061R (5′-CRRCACGAGCTGACGAC-3′). PCR conditions included initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 55 s, annealing at 54°C for 45 s, and extension at 72°C for 60 s, with final extension at 72°C for 10 min. (Andersson et. al. 2008).

The resultant PCR products were analyzed via electrophoresis on a 0.8% agarose gel containing 0.05 µg/mL ethidium bromide in 1X TAE buffer using a Gel Electrophoresis System (Genei, Mumbai). Bands were visualized and recorded with a Bio-Rad Gel Documentation system (Germany). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Germany) and outsourced to Biokart Pvt. Ltd. (India) for sequencing. The obtained sequences were analyzed using BLAST algorithm against the GenBank database. Phylogenetic analysis was conducted using MEGA X software employing the neighbor-joining method with 1000 bootstrap replications.

2.5 Probiotic Attribute Assessment

Acid Tolerance: Bacterial cultures were exposed to different pH levels (2.0, 2.5, 3.0, and 7.0) in MRS broth for 3 hours at 37°C. Survival rates were determined by comparing viable counts before and after acid exposure.

Bile Salt Tolerance: Cultures were grown in MRS broth containing different concentrations of oxgall (0.15%, 0.3%, and 0.5%) for 24 hours at 37°C. Growth was monitored by measuring optical density at 600 nm.

Cell Adhesion Assay: Adhesion to intestinal epithelial cells was evaluated using Caco-2 cell lines. Bacterial suspensions (10^8 CFU/mL) were incubated with confluent Caco-2 monolayers for 2 hours at 37°C. After washing, adherent bacteria were quantified by plate counting.

Antimicrobial Activity: The agar well diffusion method was used to assess antimicrobial activity against indicator pathogens including *E. coli* ATCC 25922, *S. aureus* ATCC 25923, and *Salmonella Typhimurium* ATCC 14028. Cell-free supernatants were tested, and inhibition zones were measured after 24 hours of incubation.

2.6 Safety Assessment

Antibiotic susceptibility was determined using the disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI 2020) guidelines. Antibiotics tested included ampicillin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), tetracycline (30 µg), and vancomycin (30 µg).

2.7 Biochemical Properties

Lactic acid production was quantified using high-performance liquid chromatography (HPLC) after 48 hours of fermentation in MRS broth. β-galactosidase activity was determined using the o-nitrophenyl-β-D-galactopyranoside (ONPG) method.

2.8 Statistical Analysis

All experiments were performed in triplicate, and the results were presented as mean ± standard deviation. Statistical analysis was performed using SPSS version 25.0, with significance level set at $p < 0.05$, using one-way ANOVA followed by Tukey's post-hoc test.

3. Results

3.1 Bacterial isolation and identification

A total of 15 LAB isolates were obtained from 42 goat faecal samples. Based on preliminary morphological and biochemical screening, five isolates showed characteristics typical of *Weissella* species. Two isolate, designated as goat faecal-7 (GF-7) and goat faecal-7 (GF-8), were selected for detailed molecular characterization based on its distinct phenotypic properties. The GF-7 and GF-8 isolates appeared as small, white, circular colonies on MRS agar with smooth margins. Microscopic examination revealed Gram-positive, rod-shaped bacteria occurring singly or in short chains. The isolates was catalase-negative and produced gas from glucose fermentation, confirming its heterofermentative nature.

3.2 Molecular identification and phylogenetic analysis

The profiles resulting from PCR amplification facilitated accurate classification of the isolates. A single band of 265 base pairs (bp) was evident in the amplified 16S rRNA product post-PCR amplification (Fig. 1). Following successful amplification, the PCR amplicon underwent forward and reverse DNA sequencing reactions, utilizing the 8F and 1492R primers, and employing the BDT v3.1 Cycle Sequencing kit along with the ABI 3730xl Genetic Analyzer. The sequences were deposited in GenBank under accession numbers MZ314055.1 (GF-7) and MZ314056.1 (GF-8).

Phylogenetic analysis confirmed the taxonomic positions of isolate GF-7 and GF-8 within the *Weissella* genus, clustering closely with other *W. confusa* strains with strong bootstrap support (98%). According to BLAST results MZ314055.1 & MZ314056.1 (*Weissella confusa*) showed 100% homology with MZ424229.1 (*Weissella cibaria*, Spain), MW450435.1 (*Weissella cibaria*, China), MZ853315.1 (*Weissella confusa*, China), MZ853347.1 (*Weissella confusa*, China) and JQ754447.1 (*Weissella confusa*, Uganda) (Fig 2).

3.3 Probiotic attributes

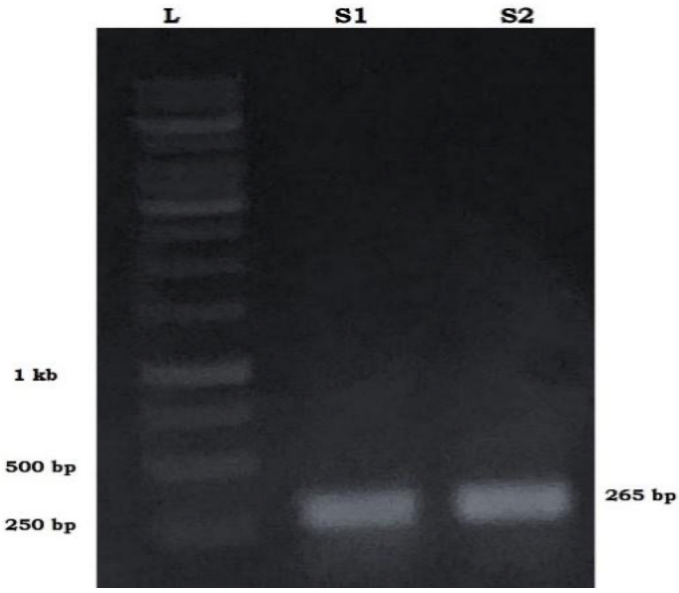

Fig. 1. *16S Amplification products of isolates*
L: DNA Ladder (2 kb)
S1: Test Sample G7
S2: Test Sample G8

Acid Tolerance: *W. confusa* GF-7 and GF-8 demonstrated significant acid tolerance with survival rates of 78.3 ± 5.2% at pH 2.5, 89.4 ± 3.7% at pH 3.0, and 95.8 ± 2.1% at pH 7.0 after 3 hours of exposure (Table 1). No viable cells were recovered at pH 2.0.

Bile Salt Tolerance: Both isolates showed good tolerance to bile salts, maintaining growth at 0.15% and 0.3% oxgall concentrations. Growth was significantly reduced at 0.5% oxgall for both GF-7 and GF-8 isolates (Fig 3).

Cell Adhesion: The adhesion assays revealed that *W. confusa* GF-7 and GF-8 demonstrated moderate adhesion to Caco-2 cells, with adhesion percentages of 34.2 ± 4.8% and 32.8 ± 3.9%, respectively, which are considered adequate for probiotic applications.

Antimicrobial Activity: Both isolates exhibited antimicrobial activity against all tested pathogenic bacteria. The inhibition zones were 18.5 ± 1.2 mm against *E. coli*, 16.8 ± 1.5 mm against *S. aureus*, and 14.2 ± 1.8 mm against *S. Typhimurium* (Table 2).

3.4. Biochemical properties

Both isolates produced significant amounts of lactic acid (GF-7: 8.4 ± 0.6 g/L; GF-8: 8.1 ± 0.7 g/L) after 48 hours of fermentation in MRS broth. β-galactosidase activity was positive in both strains, with enzyme activities of 156 ± 12 U/mg protein (GF-7) and 148 ± 15 U/mg protein (GF-8), indicating potential role in lactose metabolism (Fig. 4).

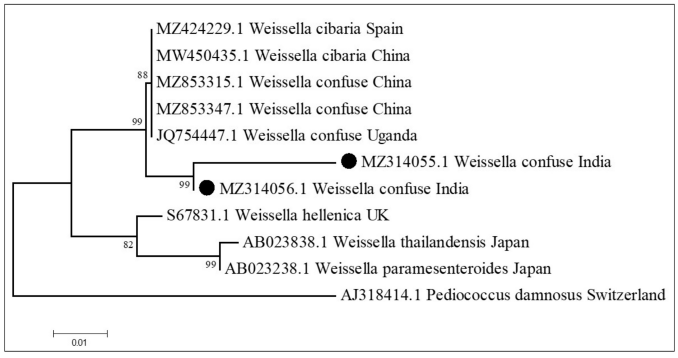

Fig. 2. *Phylogenetic analysis of isolates based on 16S rRNA sequence*

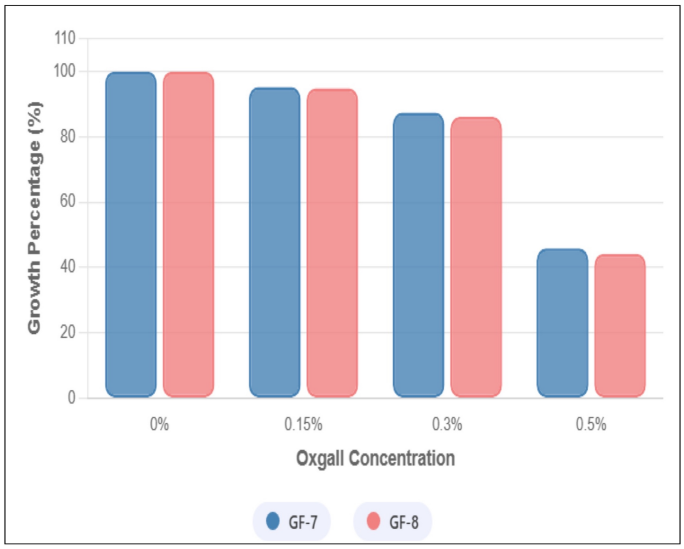

Fig. 3. *Bile salt tolerance of GF-7 and GF-8 isolates*

3.5 Safety assessment

Antibiotic susceptibility testing revealed that *W. confusa* GF-7 and GF-8 were sensitive to ampicillin, chloramphenicol, and tetracycline, showed intermediate resistance to erythromycin, and were resistance to gentamicin and vancomycin (Table 3). This resistance pattern is consistent with intrinsic resistance commonly observed in *Weissella* species.

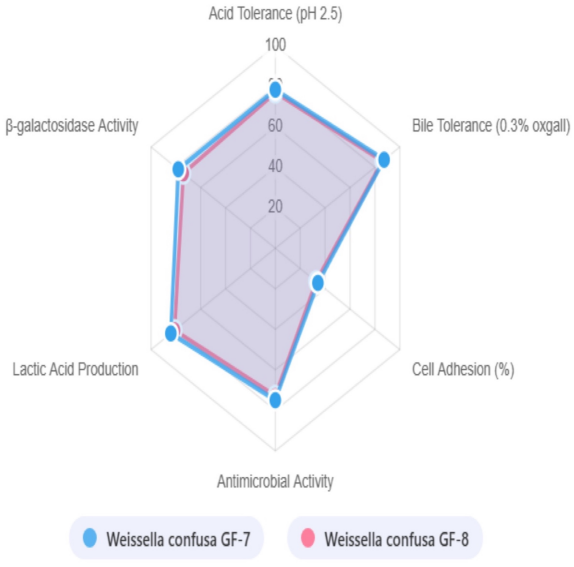

Fig. 4. *Comparative probiotic properties of GR-7 and GF-8 isolates*

4. Discussion

This study successfully isolated and characterized *W. confusa* from goat

Table 1. Acid tolerance of *Weissella confusa* isolates GF-7 and GF-8

pH	Isolate	Initial count (log CFU/mL)	Final count (log CFU/mL)	Survival rate (%)
2.0	GF-7	8.45 ± 0.12	0	0
	GF-8	8.38 ± 0.15	0	0
2.5	GF-7	8.52 ± 0.08	6.68 ± 0.24	78.3 ± 5.2
	GF-8	8.47 ± 0.11	6.52 ± 0.28	77.0 ± 4.8
3.0	GF-7	8.48 ± 0.15	7.58 ± 0.18	89.4 ± 3.7
	GF-8	8.41 ± 0.13	7.45 ± 0.21	88.6 ± 4.1
7.0	GF-7	8.41 ± 0.11	8.05 ± 0.09	95.8 ± 2.1
	GF-8	8.44 ± 0.09	8.11 ± 0.12	96.1 ± 1.8

Values are means ± standard deviation (n=3)

faeces, demonstrating its potential as a probiotic candidate. The molecular identification using 16S rRNA gene sequencing provided a definitive taxonomic classification with 99.2% similarity to the type strain, confirming the reliability of this molecular approach for bacterial identification (Stackebrandt and Ebers 2006). The ability of *W. confusa* strains GF-7 and GF-8 to withstand acidic conditions is a critical determinant of their probiotic potential, as successful passage through the gastric environment is necessary for colonization of the intestinal tract. The observed survival rate of 78.3% at pH 2.5 exceeds the minimum benchmark of 70% generally considered acceptable for

Table 2. Antimicrobial activity of *Weissella confusa* isolates GF-7 and GF-8

Test organism	Isolate	Inhibition zone (mm)	Activity level
Escherichia coli ATCC 25922	GF-7	18.5 ± 1.2	Strong
	GF-8	17.8 ± 1.5	Strong
Staphylococcus aureus ATCC 25923	GF-7	16.8 ± 1.5	Moderate
	GF-8	16.2 ± 1.8	Moderate
Salmonella Typhimurium ATCC 14028	GF-7	14.2 ± 1.8	Moderate
	GF-8	13.8 ± 2.1	Moderate
Listeria monocytogenes ATCC 19115	GF-7	12.5 ± 2.1	Weak
	GF-8	11.9 ± 1.9	Weak

Values are means ± standard deviation (n=3)

probiotic strains (Dunne et al. 2001). Such acid resistance may be facilitated by multiple mechanisms, including the induction of acid tolerance response systems, regulation of proton efflux pumps, and maintenance of intracellular pH homeostasis, which collectively enhance bacterial survival under gastric stress.

Similarly, tolerance to bile salts constitutes another prerequisite for probiotic functionality, as bile acids exert detergent-like effects on bacterial cell membranes that can lead to lysis and loss of viability. The capacity of GF-7 and GF-8 to proliferate in the presence of 0.3% oxgall indicates substantial bile resistance, consistent with physiological bile concentrations encountered in the human small intestine (Begley et al. 2005). This attribute suggests that the isolates possess intrinsic protective mechanisms such as bile salt hydrolase activity or membrane modifications that mitigate bile-induced toxicity. Adhesion to intestinal epithelial cells represents an important functional trait contributing to transient colonization, modulation of host–microbe interactions, and competitive exclusion of enteric pathogens. The adhesion efficiency of 34.2% to Caco-2 cells observed in this study indicates a moderate but functionally relevant colonization potential. Adhesion is likely mediated by surface-associated proteins, exopolysaccharides, and lipoteichoic acids, as previously reported for LAB (Tuomola et al. 2001). These adhesion levels are comparable to those described for other probiotic *Weissella* strains (Ahmed et al. 2022), thereby supporting the probiotic candidacy of GF-7 and GF-8. In addition, antimicrobial activity against pathogenic bacteria constitutes a desirable probiotic characteristic, contributing to intestinal homeostasis through inhibition of opportunistic pathogens. The broad-spectrum antagonistic effects observed in GF-7 and GF-8 may be attributable to the secretion of

Table 3. Antibiotic susceptibility of *Weissella confusa* isolates GF-7 and GF-8

Antibiotic	GF-7 Zone diameter (mm)	GF-8 Zone diameter (mm)	Interpretation
Ampicillin (10 µg)	24.5 ± 1.8	23.8 ± 2.1	Sensitive
Chloramphenicol (30 µg)	22.8 ± 2.1	22.1 ± 1.9	Sensitive
Erythromycin (15 µg)	18.2 ± 1.5	17.8 ± 1.7	Intermediate
Gentamicin (10 µg)	8.5 ± 1.2	8.9 ± 1.4	Resistant
Tetracycline (30 µg)	26.1 ± 1.9	25.4 ± 2.2	Sensitive
Vancomycin (30 µg)	9.8 ± 1.4	9.2 ± 1.6	Resistant

Values are means ± standard deviation (n=3)

organic acids, bacteriocins, and other inhibitory metabolites. Notably, the isolates demonstrated substantial lactic acid production (8.4 g/L), which not only contributes to acidification of the surrounding environment but also enhances antimicrobial efficacy, reflecting a strong fermentative capacity (Silva et al. 2020).

The antibiotic susceptibility pattern of *W. confusa* GF-7 and GF-8 requires careful consideration from a safety perspective. While sensitivity to ampicillin, chloramphenicol, and tetracycline is favorable, the resistance to gentamicin and vancomycin is concerning. However, this resistance pattern is consistent with intrinsic properties of LAB and may not pose significant safety risks because of its non-transferable nature (Danielsen and Wind 2003). However, the absence of acquired resistance genes would need to be confirmed through molecular analysis. The positive β-galactosidase activity suggests potential for

lactose metabolism, which could be beneficial for individuals with lactose intolerance. This enzyme activity enables the breakdown of lactose into glucose and galactose, potentially alleviating symptoms associated with lactose maldigestion (Savaiano and Hutkins 2021). The isolation of *W. confusa* from goat faeces highlights the potential of ruminant gut microbiota as a source of probiotic bacteria. Goats possess a diverse microbial ecosystem adapted to various dietary conditions, which may harbor strains with unique probiotic properties. The heterogeneous diet and harsh environmental conditions that goats often face may select for robust bacterial strains with enhanced stress tolerance.

Comparative analysis with other *W. confusa* strains from different sources reveals similar probiotic characteristics, suggesting species-specific traits that are conserved across different isolates. However, strain-specific variations in probiotic properties emphasize the importance of individual strain characterization for commercial applications. The technological properties of *W. confusa* GF-7 and GF-8, including lactic acid production and enzymatic activities, suggest potential applications in food fermentation and functional food development. The strain could be utilized for the production of fermented dairy products or as a direct-fed microbial supplement for livestock. Future research should focus on *in vivo* studies to evaluate the probiotic efficacy of *W. confusa* GF-7 and GF-8 in animal models. Additionally, genomic analysis would provide insights into the genetic basis of probiotic properties and safety assessment, including the absence of virulence factors and transferable antibiotic resistance genes.

5. Conclusion

This study successfully isolated and characterized *W. confusa* GF-7 and GF-8 from goat faeces, demonstrating significant probiotic potential based on molecular identification and functional assessment. The strain exhibited excellent acid and bile tolerance, moderate adhesion to intestinal epithelial cells, broad-spectrum antimicrobial activity, good fermentative capacity, and lactose metabolism potential. While some concerns exist regarding antibiotic resistance, the overall safety profile appears acceptable for probiotic applications. The findings contribute to the understanding of *Weissella* species diversity and their potential as probiotic candidates. *W. confusa* GF-7 and GF-8 represents a promising strain for further development as a probiotic supplement for both human and animal applications, pending additional safety evaluations and efficacy studies.

Declarations

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Conflict of interest: Authors declare no conflicts of interest

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