Isolation and characterization of lactic acid bacteria from traditional pickles of Himachal Pradesh, India

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Abstract A total of 15 isolates of lactic acid bacteria (LAB) were isolated from traditional pickles of rural and urban areas of Himachal Pradesh. These isolates were identified as *Enterococcus faecalis* (7), *Lactobacillus plantarum* (3), *Pediococcus pentosaceus* (2), *Leuconostoc mesenteroides* (1), *Lactococcus lactis* (1) and *Enterococcus* sp. (1) on the basis of morphological, biochemical and 16S rDNA sequence analysis. All the LABs produced exopolysaccharides, have antibiotic susceptibility, and possess activity of β -galactosidase, β -glucosidase, protease and amylase, while none of the isolates showed haemolytic activity. Out of 15 isolates tested, only 6 isolates showed the phytase activity. These LAB exhibited antimicrobial activity against food borne pathogenic bacteria i.e. *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus* and *Shigella dysenteriae*. These results suggested that LAB isolates from traditional pickles of Himachal Pradesh have very good potential to be used as probiotics.

Keywords Lactic acid bacteria · Himachal Pradesh · Pickles · Probiotic · Traditional

Introduction

Pickling is one of the oldest methods of food preservation by fermentation. It is the process of conversion of sugars to acids by microorganisms (Nurul and Asmah 2012). Pickles are good appetizers, consumed by people of all ages and

contain large amounts of lactobacilli which are important for the digestion of grains and vegetables, and have beneficial probiotic properties (Lal et al. 2010). Pickles prepared by lactic acid bacteria (LAB) fermentation have unique flavour and beneficial health effects (Choi et al. 2013). LAB in pickles help to enhance human nutrition by providing vitamins, minerals, and carbohydrates, and produce various aroma components, bacteriocins, and exopolysaccharides. These metabolic products impart some characteristic properties such as taste, texture and longer shelf life to the products (Leroy and de Vuyst 2004). LAB carry out detoxification of toxic compounds and degradation of mycotoxins in specific cases and therefore can reduce the health risk. Lactic acid bacteria are considered as safe additives and Generally Recognized As Safe (GRAS), useful to control the frequent development of pathogens and spoilage microorganisms in food and feed (Namasivayam et al. 2014).

A variety of ingredients such as salt, vinegar, spices viz. chilli powder, turmeric powder, fenugreek seed, asafoetida, sugar, jaggery and oil are frequently used in pickling in different parts of the world. In India, the pickles vary in terms of spices and method used for their preparation e.g. black mustard is a unique ingredient, which gives characteristic traditional flavor to the pickles made in Himachal Pradesh that make these different from the rest of the states in India. However, it is not used in the pickles prepared from ingredients already having acidic taste/sour taste (Monika et al. 2016). The microorganisms involved in the fermentation of pickles have been identified as *Lactobacillus plantarum*, *L. brevis*, *Leuconostoc mesenteroides* and *Pediococcus cerevisiae*, *Pediococcus pentosaceus* and *Enterococcus faecalis* (Choi et al. 2013; Jagannath et al. 2012). The LAB isolated from pickles differ from other

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microorganisms as they can tolerate considerably high concentration of salt and sugar.

There is no information about the microflora of traditional pickles of Himachal Pradesh. Keeping this in view, the aim of present study was to isolate and identify lactic acid bacteria from traditional pickles of Himachal Pradesh and screen them for antimicrobial activity against food borne pathogens. Further, antibiotic susceptibility and various enzymatic activities were studied in order to use LAB as potential candidate for probiotic application.

Materials and methods

Collection of samples

Samples of traditional pickles used for the isolation of LAB were collected from different places of rural and urban areas of Himachal Pradesh, India. The pickles were collected in clean polythene bags and brought to laboratory and stored in refrigerator till further analysis. Different food borne pathogenic test microorganisms i.e. *Escherichia coli*, *Shigella dysenteriae*, *Staphylococcus aureus* and *Bacillus cereus* were procured from the Department of Biotechnology, Himachal Pradesh University, Shimla, India.

Isolation of lactic acid bacteria

Lactic acid bacteria (LAB) were isolated from traditional pickles by appropriate dilutions with saline, plating on MRS (de Mann Rogosa Sharpe) agar supplemented with 1% CaCO_3 to distinguish the acid-producing bacteria from other bacteria and then incubating anaerobically at 30 °C for 48 h. Colonies of acid-producing bacteria, identified by a clear zone around each colony, were randomly selected from MRS agar plates and purified by replating on MRS agar. Colonies were selected on the basis of gram staining, catalase test and absence of spores and were preserved in 40% glycerol at −80 °C.

Physiological and biochemical characterization

The isolates were further characterized on the basis of biochemical tests i.e. carbohydrate fermentation, tolerance to NaCl at concentration of 1.5–10%, growth at different pH i.e. 3.5, 6.5 and 8.5, growth at different temperature i.e. 15, 30 and 45 °C, citrate utilization and arginine hydrolysis.

DNA extraction and 16S rDNA sequencing of LAB isolates

Chromosomal DNA of LAB isolates were extracted by using the methods of Sambrook et al. (1989). PCR amplifications

was done by using universal primer for 16S rDNA under following conditions: 5 min at 95 °C, 30 cycles of 40 s at 95 °C, 60 s at 53 °C, 2 min at 72 °C and final extension at 72 °C for 5 min. Amplified PCR product of LAB isolates were sequenced at Xcelris Genomics, Ahmadabad, India. Nucleotide sequences were analyzed for similarities in NCBI by BLAST and submitted to GenBank. Related sequences were obtained from NCBI, aligned by ClustalW and a phylogenetic tree was constructed using Mega 6.06 software.

Antimicrobial activity

Antimicrobial activity of LAB were analyzed by using agar well diffusion method (Yang et al.1992). The indicator strains viz. *E. coli*, *B. cereus*, *S. aureus* and *S. dysenteriae* were used.

Antibiotic susceptibility

The antibiotic susceptibility of LAB isolates was done by using antibiotic discs containing penicillin (P) 10 units, erythromycin (E) 15 µg, vancomycin (VA) 30 µg, teicoplanin (TE1) 30 µg, clindamycin (CD) 2 µg, ofloxacin (OF) 5 µg, azithromycin (AZM) 15 µg and tetracycline (TE) 30 µg. MRS agar plates were spread with 100 µl of an overnight LAB culture and antibiotic discs were placed under sterile conditions. After incubation for 24 h at 30 °C, the diameter (mm) of inhibition zone was measured and results were expressed as resistant and susceptible.

Exopolysaccharide production

Overnight grown LAB cultures were streaked on the surface of plates containing skim milk powder (10% w/v), sucrose (1% w/v), ruthenium red (0.08 g/l), agar (1.5% w/v) and these were incubated at 30 °C for 24 h. The non ropy strains gave red colonies due to the staining of the bacterial cell wall and ropy strains appeared as white colonies.

Haemolytic activity

Overnight grown cultures were streaked on the surface of Sheep blood agar (HiMedia, Mumbai, India) and incubated at 30 °C for 24 h. The haemolytic reaction was recorded by observation of a partial hydrolysis of red blood cells and greening zone (α -haemolysis), clear zone around bacterial growth (β haemolysis) and no reaction (γ -haemolysis).

β -Galactosidase activity

Bacterial cultures were streaked on MRS agar plates containing 60 µl of 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) and 40 µl of 1 mg/ml IPTG

(isopropyl β -D-thiogalactopyranoside) solution as inducer. The appearance of the blue colonies on MRS agar indicated the presence of β -galactosidase activity. The quantitative screening of β -galactosidase activity was determined by using 4 mg/ml of o-nitrophenol- β -D-galactopyranoside (o-NPG) in 0.1 M sodium phosphate buffer (pH 6.8). The reaction course was monitored for 10 min, by measuring the concentration of released o-nitrophenol (o-NP) spectrophotometrically at 410 nm (Miller 1972). One unit (IU) is defined as the amount of the enzyme that catalyzes the liberation of 1 μ mol o-NP per min under the specified assay conditions.

Phytase activity

The phytase activity of LAB isolates was detected using method described by Bae et al. (1999). To eliminate false positive results caused by microbial acid production, the petri plates were flooded with 2% (w/v) aqueous cobalt chloride solution. After 5 min of incubation at room temperature, the cobalt chloride solution was removed and then freshly prepared solution containing equal volumes of a 6.25% (w/v) aqueous ammonium molybdate solution and 0.42% (w/v) ammonium vanadate solution were added. Following 5 min of incubation, the ammonium molybdate/ammonium vanadate solution was removed and the plate was examined for zones of phytate hydrolysis.

Proteolytic activity

Primary screening of LAB isolates for their proteolytic activity on skim milk agar plate was done according to Gordon et al. (1973). Secondary (quantitative) screening was done according to Manachini et al. (1988).

β -Glucosidase activity

β -Glucosidase activity was determined according to the method described by Matsuda et al. (1994) using p-nitrophenyl- β -D-glucopyranoside as substrate.

Amylase activity

Amylase activity was determined by employing the 3,5-dinitrosalicylic acid (DNS) as per the method suggested by Bernfeld (1955).

Result and discussion

Isolation and identification of bacterial isolates

A total of 15 lactic acid bacterial isolates were isolated from traditional pickles of Himachal Pradesh. These

isolates were identified on the basis of physiological and biochemical characteristics presented in Tables 1 and 2. These isolates were further identified on the basis of 16S rDNA sequencing as *Enterococcus faecalis* (PKL-3, PKL-4, PKL-7, PKL-24, PKL-25, PKL-27 and PKL-34), *Lactobacillus plantarum* (PKL-15, PKL-20 and PKL-21), *Leuconostoc mesenteroides* (PKL-6), *Lactococcus lactis* (PKL-5), *Pediococcus pentosaceus* (PKL-14 and PKL-17) and *Enterococcus* sp. (PKL-28). Among them *Enterococcus faecalis* is dominant species. The 16S rDNA gene sequences of LAB isolates have been submitted to the GenBank databases under accession numbers KR137532 to KR137544 and KT124547 and KT124549 (Table 3). Phylogenetic relationship between LAB was constructed based on the 16S rDNA sequences from evolutionary distances by the neighbor-joining method (Fig. 1). Lactic acid bacteria were clustered in three groups i.e. *Enterococcus*, *Pediococcus* and *Lactobacillus* while *Lactococcus* showed divergence from other *Lactobacilli*. Earlier, Kawahara et al. (2010) isolated *Lactobacillus curvatus* strain Y108 from traditional Japanese pickle *Nozawana-zuke*. In a study Ozkalp et al. (2009) the lactic acid bacteria were isolated from caper pickle. Among them *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus fermentum* and *Lactobacillus brevis* were dominant species.

Antimicrobial activity

A wide spectrum of antimicrobial activity was observed for LAB isolates against *E. coli*, *S. aureus*, *B. cereus* and *S. dysenteriae* by agar well diffusion assay method. Most of the isolates inhibited the growth of *E. coli*, *S. aureus*, *B. cereus* and *S. dysenteriae*. As shown in Fig. 2, all the isolates were active against *E. coli* and *B. cereus*. However, *L. plantarum* (PKL-21) showed wide inhibitory spectrum against all the gram positive and gram negative bacteria i.e. *B. cereus* (16.36 mm), *E. coli* (13.1 mm), *S. dysenteriae* (12.86 mm), *S. aureus* (8.3 mm). *E. faecalis* (PKL-34) did not show antimicrobial activity against *S. dysenteriae* and *L. plantarum* (PKL-20), *E. faecalis* (PKL-25) and *Enterococcus* sp. (PKL-28) did not show activity against *S. aureus*. The antimicrobial activity of LAB may be due to a number of factors like decrease in pH level, competition for substrates and the production of substances (e.g. bacteriocin) with a bactericidal or bacteriostatic action (Pariente and Ricciardi 1999). In fact, the drop in pH arising from the production of lactic acid can be enough to inhibit certain strains.

Antibiotic susceptibility

Antibiotic susceptibility of LAB is one of the important criteria for the safety of probiotics. All the LAB isolates

Table 1 Physiological and biochemical characteristics of lactic acid bacteria isolated from traditional pickles of Himachal Pradesh

LAB isolates	Source	Botanical name	Cell shape	Catalase test	Endospore test	Carbohydrate fermentation					
						Sucrose	Lactose	Maltose	Mannitol	Dextrose	Starch
PKL-3	Aaroo	<i>Prunus persica</i>	Cocci	—	—	+++	+++	+++	+	+++	—
PKL-4	Aaroo	<i>Prunus persica</i>	Cocci	—	—	+++	+++	+++	++	+++	++
PKL-5	Mirch	<i>Capsicum annum</i>	Cocci	—	—	+++	+++	+++	++	+++	++
PKL-6	Galgol	<i>Citrus psedolimon</i>	Cocci	—	—	+++	+++	+++	+	+++	—
PKL-7	Galgol	<i>Citrus psedolimon</i>	Cocci	—	—	+++	+++	+++	+	+++	—
PKL-14	Japani	<i>Diospyros kaki</i>	Cocci	—	—	+++	+++	+++	+	+++	+
PKL-15	Lingri	<i>Diplazium esculentum</i>	Rod	—	—	+++	+++	+++	++	+++	+
PKL-17	Nimbu	<i>Citrus limon</i>	Cocci	—	—	+++	+++	+++	++	+++	+
PKL-20	Aam	<i>Mangifera indica</i>	Rod	—	—	+++	+++	+++	+	+++	+
PKL-21	Aam	<i>Mangifera indica</i>	Rod	—	—	+++	+++	+++	++	+++	+
PKL-24	Lingri	<i>Diplazium esculentum</i>	Cocci	—	—	+++	+++	+++	++	+++	+
PKL-25	Lingri	<i>Diplazium esculentum</i>	Cocci	—	—	+++	+++	+++	+++	+++	+
PKL-27	Lingri	<i>Diplazium esculentum</i>	Cocci	—	—	+++	+++	+++	++	+++	+
PKL-28	Plum	<i>Prunus domestica</i>	Cocci	—	—	+++	+++	+++	++	+++	+
PKL-34	Mirch	<i>Capsicum annum</i>	Cocci	—	—	+++	+++	+++	+++	+++	+

+, minimum growth; ++, moderate growth; +++, maximum growth; —, no growth

were found to be susceptible to all the antibiotics tested except vancomycin and teicoplanin. Out of fifteen isolates, four isolates were resistant to vancomycin i.e. (*L. plantarum* (PKL-15, PKL-20), *E. faecalis* (PKL-25, PKL-27), *Enterococcus* sp. (PKL-28) and three isolates were resistant to teicoplanin i.e. *E. faecalis* (PKL-25, PKL-27) and *Enterococcus* sp. (PKL-28). The resistance to vancomycin observed in this study was in accordance with other reported study (Zhou et al. 2005). An important drawback of antibiotic resistance is that transfer of antibiotic resistance gene is possible. Because antibiotic resistant genes are generally carried on plasmids, they can be transferred to other bacteria by means of conjugation. This resistance is due to the presence of D-Ala-D-Lactate in their peptidoglycan rather than D-Ala-D-Ala dipeptide which is the target of the antibiotic (Danielsen and Wind 2003). There is a risk associated with the ability of these resistant strains to transmit the resistance gene to pathogenic bacteria (Mathur and Singh 2005). This may result to highly antibiotic resistant enteropathogenic bacteria.

Exopolysaccharide (EPS) production

Exopolysaccharides (EPS) are the major component of the bacterial biofilm which enhances the colonization of probiotic bacteria by cell interaction in gastrointestinal tract and in the protection of bacteria against lethal influence of

the environment (Kanmani et al. 2013). All the LAB isolates produced EPS and form slimy white ropy colonies on skimmed milk ruthenium agar which is in consistency with the earlier study (Dabour et al. 2006). Ruthenium red stains the bacterial cell wall, producing pink colonies from non ropy strains. In ropy strains, EPS prevents uptake of the stain and the colonies appear white (Stingele and Mollet 1995).

Haemolytic activity

Absence of haemolytic activity is considered as a safety prerequisite for the selection of a probiotic strain. None of the LAB isolates showed α and β haemolytic activity when grown on Columbia sheep blood agar. All the LAB isolates were γ haemolytic (no haemolysis). Similar observations were made for the strains of *L. paracasei* subsp. *paracasei*, *Lactobacillus* spp. and *L. casei* isolated from the dairy products which showed γ haemolysis (Maragkoudakis et al. 2006). Most of the LAB isolated from foods have been reported to be γ haemolytic (Kumar and Murugalatha 2012).

β -Galactosidase activity

β -Galactosidase activity is an essential feature in probiotic organisms. Its deficiency (lactose intolerance) is linked to

Table 2 Phenotypic properties of lactic acid bacteria isolated from traditional pickles of Himachal Pradesh

Isolates	pH			NaCl						Temperature			Citrate utilization			Arginine hydrolysis
	3.5	6.5	8.5	1.5%	2.5%	5%	6.5%	8.5%	10%	15 °C	30 °C	45 °C	Gas formation	Colour changes	Growth	
PKL-3	–	+	+	+++	+++	+++	++	+	–	+	+	–	+	–	+++	+
PKL-4	–	+	+	+++	+++	++	++	+	–	+	+	–	–	–	+++	+
PKL-5	–	+	+	+++	++	–	–	–	–	+	+	–	–	–	–	+
PKL-6	+	+	+	+++	+++	++	++	–	–	+	+	+	++	–	+++	–
PKL-7	–	+	+	+++	+++	++	+	–	–	+	+	+	–	–	–	+
PKL-14	–	+	+	+++	+++	++	+	–	–	+	+	+	–	–	–	+
PKL-15	+	+	+	+++	+	–	–	–	–	+	+	–	+++	–	+++	+
PKL-17	+	+	+	+++	++	++	+	–	–	+	+	+	+	–	+	+
PKL-20	+	+	+	+++	+++	++	++	–	–	+	+	–	–	–	+++	+
PKL-21	+	+	+	+	+	–	–	–	–	+	+	–	+++	–	+++	–
PKL-24	–	+	+	+++	++	++	++	–	–	+	+	+	–	–	–	–
PKL-25	–	+	+	+++	+++	–	–	–	–	+	+	+	–	–	–	+
PKL-27	–	+	+	++	++	+	–	–	–	+	+	+	–	–	–	+
PKL-28	+	+	+	+++	+++	–	–	–	–	+	+	–	–	–	–	+
PKL-34	+	+	+	+++	++	++	+	+	–	+	+	–	+	–	+++	+

+, minimum growth; ++, moderate growth; +++, maximum growth; –, no growth

Table 3 List of lactic acid bacterial isolates identified on the basis of 16S rDNA sequence with NCBI accession numbers

Strain	Organism name	Accession number
PKL-3	<i>Enterococcus faecalis</i>	KR137534
PKL-4	<i>Enterococcus faecalis</i>	KR137540
PKL-5	<i>Lactococcus lactis</i>	KT124547
PKL-6	<i>Leuconostoc mesenteroides</i>	KR137536
PKL-7	<i>Enterococcus faecalis</i>	KR137541
PKL-14	<i>Pediococcus pentosaceus</i>	KR137539
PKL-15	<i>Lactobacillus plantarum</i>	KT124549
PKL-17	<i>Pediococcus pentosaceus</i>	KR137533
PKL-20	<i>Lactobacillus plantarum</i>	KR137535
PKL-21	<i>Lactobacillus plantarum</i>	KR137542
PKL-24	<i>Enterococcus faecalis</i>	KR137543
PKL-25	<i>Enterococcus faecalis</i>	KR137544
PKL-27	<i>Enterococcus faecalis</i>	KR137532
PKL-28	<i>Enterococcus sp.</i>	KR137537
PKL-34	<i>Enterococcus faecalis</i>	KR137538

the inability to breakdown lactose in the duodenum (Moser and Savage 2001). In plating method, all the 15 LAB isolates produced blue coloured colonies on X-gal plates indicating the presence of β -galactosidase enzyme. In quantitative screening, twelve isolates possessed the presence of β -galactosidase having specific activity in the range of 0.01–0.07 U/mg dcw (Fig. 3a). In the present study, all

the LAB isolates possessed β -galactosidase activity with the maximum specific activity (0.07U/mg dcw) in the isolate *P. pentosaceus* PKL-17.

Phytase activity

Phytases are considered to be useful in upgrading the nutritional quality of phytate-rich foods and feeds. Phytate acts as an antinutrient by binding to proteins and by chelating minerals (Cheryan 1980), the addition of phytase can improve the nutritional value of plant based foods by enhancing protein digestibility and mineral availability through phytate hydrolysis during digestion in the stomach or during food processing (Sandberg and Andlid 2002). Out of fifteen LAB isolates screened for phytase activity, six isolates showed clear zones for phytase activity. Highest activity was observed in *L. plantarum* PKL-21 (22 mm) followed by *P. pentosaceus* PKL-17 (16 mm), *Enterococcus sp.* PKL-28 (8 mm), *P. pentosaceus* PKL-14 (7 mm), *L. plantarum* PKL-20 (6 mm) and the lowest activity was observed in *L. plantarum* PKL-15 (5 mm) which counter-parts with the study of Sumengen et al. (2012).

Proteolytic activity

In quantitative screening of selected fifteen LAB isolates, fourteen isolates showed protease activity with specific

Fig. 1 Phylogenetic tree showing the relative position of *Leuconostoc mesenteroides*, *Lactococcus lactis*, *Lactobacillus plantarum*, *Pediococcus pentosaceus*, *Enterococcus* sp. and *Enterococcus faecalis* as inferred by the neighbor-joining method with 16S rRNA gene sequences. *Bordetella pertussis* is used as an outgroup. The bar indicates 2% sequence divergence

Fig. 2 Antimicrobial activity of lactic acid bacteria isolated from traditional pickles of Himachal Pradesh against food borne pathogen by using well diffusion method

activity ranging 0.11–3.64 U/mg protein. Highest activity was measured in *E. faecalis* PKL-3 (3.64 U/mg protein) followed by *E. faecalis* PKL-24 (3.35 U/mg protein) and lowest activity was observed in *E. faecalis* PKL-27 (0.11 U/mg protein) as shown in (Fig. 3b). The proteolytic ability of lactic acid bacteria depends on species and strain. The proteolytic enzymes of *L. bulgaricus* degraded casein with the liberation of low molecular weight peptides and amino acids (Rajagopal and Sandine 1990).

β-Glucosidase activity

All the LAB isolates showed β-glucosidase activity ranging from 0.01 to 1.39 U/mg dcw. The isolate *P. pentosaceus* PKL-17 (1.39 U/mg dcw) showed maximum activity and lowest activity was observed in *E. faecalis* PKL-25 (0.01

U/mg dcw) as shown in (Fig. 3c). β-Glucosidase catalyses the hydrolysis of glucoside isoflavones with the formation of aglycones (Esaki et al. 2004). The intestinal microflora such as *Lactobacillus* and *Bifidobacterium* can produce β-glucosidase that plays a key role in the metabolism and bioavailability of isoflavones, as they are able to produce β-glucosidase (Setchell 2000).

Amylase activity

The α-amylase activity of LAB isolates is shown in (Fig. 3d). Highest activity was observed in *E. faecalis* PKL-25 (1.75 U/mg protein) followed by *P. pentosaceus* PKL-17 (1.16 U/mg protein) and lowest activity was observed in *E. faecalis* PKL-3 (0.02 U/mg protein). Amylolytic LAB utilize starchy biomass and convert it into

Fig. 3 **a** β -Galactosidase activity of LAB isolated from traditional pickles of Himachal Pradesh. **b** Protease activity of LAB isolated from traditional pickles of Himachal Pradesh. **c** β -Glucosidase

activity of LAB isolated from traditional pickles of Himachal Pradesh. **d** Amylase activity of LAB isolated from traditional pickles of Himachal Pradesh

lactic acid in a single step fermentation. Amylases in LAB species was reported by Agati et al. (1998) and Petrov et al. (2008).

Conclusion

This study reported for the first time, the isolation, characterization and identification of LAB from traditional fermented pickles of Himachal Pradesh. Six different type of lactic acid bacteria were identified in traditional pickles. The most of the isolates showed zone of inhibition against food borne pathogens, antibiotic susceptibility, exopolysaccharides production, γ haemolysis and activity of useful enzymes such as β -galactosidase, phytase, β -glucosidase, protease and amylase. These LAB isolates thus have great potential to be used as probiotics.

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Compliance with ethical standards

Conflict of interest All the Authors declare that there is no conflict of interest.

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