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Probiotic characterization of lactic acid bacteria isolated from human milk

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Abstract
The objective of this study was to evaluate the probiotic properties of lactic acid bacteria isolated from human milk. Based on biochemical characterization and 16S rRNA sequencing, the isolate was identified as *Pediococcus pentosaceus* DM101, the sequence of which is deposited in NCBI with accession number MK774704. Probiotic characterization in terms of acid tolerance, bile tolerance, adhesion potential, and safety of was done in *vitro*. *Pediococcus pentosaceus* DM101 was found to withstand pH 3.0 and 0.6% (w/v) bile salt for four hours. Auto-aggregation and cell surface hydrophobicity values of 68% and 60% signifies the good adhesion potential of the isolate. Inability to cause hemolysis and liquefy gelatin suggests the possible absence of virulence factors. Antibiogram revealed the isolate to be resistant to Vancomycin, Methicillin, Bacitracin, and Cephalosporin group of antibiotics. Exopolysaccharide producing nature of the isolate was confirmed by Congo red assay. DPPH assay of the cell free supernatant revealed an IC 50 value of 20.78mg/ml, endorsing its radical scavenging potential. The results obtained in this study decipher *Pediococcus pentosaceus* DM101 as a propitious candidate for designing foods to counteract oxidative stress proactively.

Keywords: *P.pentosaceus*, breast milk, probiotic, exopolysaccharide

1. Introduction
Human breast milk is recognized as the gold standard of infant feeding (Serrano-Niño *et al;* 2016) [32]. The integral role of breast milk in the development of infants is by its nutrient composition and diverse microbiome that is unique for each mother (Martin *et al*; 2012) [21]. According to LaTuga *et al*. (2014) [17] breast milk microbiome is influenced by maternal health and nutrition, mode of delivery, stage of lactation, and breast skin microflora. Retrograde movement of microbes from the infant oral cavity to ductal tissue (Hunt *et al;* 2011) [13] and transfer from maternal skin (Perez *et al.*, 2007) [27] are also responsible for the presence of microorganisms in breast milk. Lactic Acid Bacteria (LAB) isolated from breast milk is particularly attractive in probiotic foods due to their, human origin and their presumed safety (Martin *et al*., 2004) [20]. Apart from *Lactobacillus* and *Bifidobacterium,* members of *Pediococcus, Weissella*, *Lactococcus*, and *Enterococcu*s have also been recognized for their health-promoting effects (Zommiti *et al*., 2018) [40].
There are earlier studies reporting the isolation of probiotic lactic acid bacteria from human milk (Osmanagaoglu *et al*., 2010) [24].
Oxidative stress is a condition arising from the imbalance between pro-oxidants and antioxidants that lead to protein denaturation, lipid peroxidation, DNA hydroxylation, and ultimately, interference to cell metabolism and viability. In the current lifestyle, fried foods have become an indispensable part of diet. Oxidized oil entering body systems through such unhealthy diet creates oxidative stress.
Living cells do possess enzymatic defenses like superoxide dismutase, glutathione peroxidase, and glutathione reductase in addition to non-enzymatic defenses like glutathione, thioredoxin, Vitamin C and Vitamin E against oxidative stress(Mishra *et al*., 2015) [22]. However, many times these native antioxidant systems alone will not be sufficient to afford protection from oxidative damage.
As synthetic antioxidants raise a safety concern, antioxidants of biological origin are of special interest. There are reports of lactic acid bacteria with remarkable potential to scavenge free oxygen radicals. Such bacteria have immense potential to be used for dietary intervention, as probiotics in the proactive management of lifestyle diseases associated with oxidative stress. The present study was taken up to isolate a probiotic candidate from breast milk.

2. Materials and methods

2.1 Isolation and identification of lactic acid bacteria

Aseptically collected breast milk sample, after pre-enrichment in Nutrient broth (Hi-Media) was appropriately diluted and pour plated in De Man, Rogosa, and Sharpe (MRS) agar (Hi-Media). Incubation was done at 37^0C for 48h to get well isolated discreet colonies. The typical spindle-shaped colonies were selected and streaked on MRS agar for further purification. The purified isolate was maintained in MRS agar slant at 4°C and also in 70 percent glycerol at -18^0C for long term storage.

Morphological and biochemical identification at the preliminary level was performed according to the methods described in Bergey's Manual of systematic Bacteriology for identification of LAB (Vos *et al*., 2009) [38]. All the tests for identification were carried out using freshly activated cultures in MRS broth. Molecular-level confirmation of the isolate was done by 16S rRNA sequencing. The primers used were 16S-RS-F Forward 5' CAGGCCTAACACATGCAAGTC3' and 16S-RS-R Reverse 5' GGGCGGWGTGTACAAGGC 3

2.2 Probiotic characterization

Probiotics bring about its beneficial effects by altering the intestinal microflora in a way advantageous to the consumer. The probiotic properties of the breast milk isolate in terms of acid tolerance, bile tolerance, adhesion potential, and safety were evaluated *in vitro*

2.2.1 Acid and bile tolerance

The isolate was exposed to pH 2.0 and 3.0 by inoculating into sterile MRS broth tubes whose pH was adjusted using 0.1N Hydrochloric acid. For evaluating the bile tolerance, the isolate was exposed to 0.3 and 0.6 percent bile environment by inoculating into sterile MRS broth tubes containing bile salts. Incubation was done at 37^0C. The number of survivors was qualitatively assessed by streaking on MRS agar plates at hourly intervals for four hours. (Pundir *et al*., 2013) [28].

2.2.1 Adhesion Potential

Adhesion potential was judged by determining autoaggregation percentage and cell surface hydrophobicity (CSH value).

2.2.2.1 Auto-aggregation

Autoaggregation assay was performed as per Kos *et al*. (2003) [15]. The cell pellet from freshly activated culture (37°C/18h) was suspended in phosphate buffer to give a final optical density of 0.60±0.02 at 600nm. 0.1ml of the suspension was mixed with 3.9 ml of phosphate buffered saline and the absorbance (A1) was measured at 600 nm. The sample was kept undisturbed for at 37°C to allow sedimentation. Optical density (OD) of the sample (A2) was again determined at one hour and six hours.

The auto-aggregation percentage was expressed as:

Auto-aggregation (percentage) = [(A1 - A2)/ (A1) x 100]

Where

A1: initial optical density,

A2: optical density after incubation.

Autoaggregation was calculated from three replicates as the percentage decrease in absorbance of the original suspension due to aggregation and sedimentation

2.2.2.2. Bacterial cell surface hydrophobicity

Cell-surface hydrophobicity was determined as per Collado *et al.* (2008) [5]. The cell pellet from 18 h old freshly activated cultures was suspended in phosphate buffer to give a final optical density 0.25±.05 at 600 nm. Equal volume of xylene was added to the cell suspension and vortexed thoroughly. The mixture was kept undisturbed for one hour to allow the phases to separate. After one hour, the upper phase was carefully pipetted out and OD was again determined.

$$\frac{\text{Initial OD- Final OD}}{\text{Initial OD}} \times 100$$

2.2.3 Safety assessment

Safety evaluation was done by looking at its ability to cause lysis of blood cells (Adetoye *et al*., 2018) [1] and liquefy gelatin (Sahu *et al*., 2019) [29].

2.2.3.10020Hemolytic property

The isolate was streaked on blood agar plate. Plate was examined for the presence of zones around the growth after incubation at 37°C for 24 h. The isolate that produced green-hued zones around the colonies (alpha-hemolysis) or those that did not produce any zone on the blood agar (Gamma-hemolysis) were considered as non-hemolytic. Those producing zones of clearance (Beta- hemolysis) were classified as hemolytic (Adetoye *et al*., 2018) [1].

2.2.3.2 Gelatin liquefaction

The isolate was streaked on Gelatin agar (Hi-Media) slant and incubated at 37°C for 24 h. Uninoculated tube served as control. After incubation, both tubes were kept for 3h under refrigeration before reading the result to affirm that liquefaction is due to microbial action and not due to the incubation temperature employed. Partial or total liquefaction of the inoculated tube when compared to the control tube was taken as positive for gelatin liquefaction (Sahu *et al*., 2019) [29].

2.2.3.3 Antibiogram of the isolate

Antibiogram of the isolate was evaluated by the Disc diffusion method following modified standard Kirby–Bauer procedure (Bauer *et al*., 1966) [4]. The antibiotics tested were Cefotaxime (10 mcg), Chloramphenicol (30mcg), Erythromycin (10mcg), Penicillin G (10units), Ampicillin (10mcg), Streptomycin (10mcg), Tetracyclin (30mcg), Co-Trimoxazole (25mcg), Bacitracin (30mcg), Enrofloxacin (10mcg), Cephalothin (30mcg), Azithromycin(15mcg), Ofloxacin (5mcg), Ciprofloxacin (30mcg), Linezolid(30mcg), and Gentamycin(120mcg). The zone of inhibition was measured using an antibiotic zone scale and expressed in millimeter. The isolates were categorized: zone of inhibition ≥21mm were graded as susceptibile, 16-20mm as moderately susceptible and ≤15mm as resistant (Vlkova *et al.* 2006) [37].

2.3 Functional properties

2.3.1 EPS production

EPS production potential was evaluated based on colony characteristics when streaked on Congo red agar; a sugar supplemented medium containing Congo red (Freeman *et al*., 1989) [9]. Brain Heart Infusion agar to which one percent Congo red solution was added at a level of nine percent and sucrose at five percent served as the medium. Incubation was done at 37^0C. Formation of slimy and shining black colonies

within 24h of incubation was suggestive of EPS production.

2.3.2 Anti-oxidant assay

The radical scavenging activity of cell-free supernatant (CFS) was determined by 2,2-diphenyl picryl hydrazyl(DPPH) assay as detailed by Shehata *et al*.(2019)[33].The CFS was prepared from two milliliters of freshly activated broth culture by centrifugation(Centrifuge MODEL SLM-CFT-10K, GeNei) at 5000rpm for 15minutes. Different volumes of CFS (0.5, 1.0 and 1.25ml) were added to 1.5 ml of freshly prepared 0.066mM DPPH. The mixture was vigorously shaken and left to react for 30 minutes in the dark at 37ºC. The volume was made up to 3.0 ml with methanol. The blank contained DPPH solution and methanol (1:1). The absorbance was measured at 517 nm. The scavenging ability was calculated as follows:

$$\text{Scavenging activity (\%)} = \frac{(\text{Absorbance of blank} - \text{Absorbance of the sample})}{\text{Absorbance of blank}} \times 100$$

A standard curve was plotted with the scavenging activity (%) on the Y-axis and different concentrations of the sample (ppm) on the X-axis. The antioxidant activity of the sample was expressed as the IC 50 value (mg/L or mg/ml). IC 50 value is the concentration of inhibitor required to bring about 50 percent inhibition of an enzymatic reaction at a specific substrate concentration (Paolini *et al*., 2010) [26]. The IC 50 value was calculated from the standard curve.

3. Results and discussion

3.1 Isolation and identification of lactic acid bacteria

Breast milk is a treasure house of bioactive molecules and a diverse population of commensal microflora. This study attempted to identify a probiotic candidate from aseptically collected breast milk. Pour plating of the sample in MRS agar revealed the presence of subsurface spindle-shaped colonies (Fig 1) indicating their aerotolerant nature (Vos *et al*.,2009) [38]. Though diverse microflora is reported in breast milk (Sinkiewicz and Nordström, 2005) [36] in the experimental conditions followed in this study, only one type of colony morphology was evident. Remarkable sliminess was evident for the colonies obtained by streaking on MRS agar.

The isolate obtained from breast milk was Gram-positive, cocci that was catalase and oxidase negative and arranged as tetrads or diplococcic (Fig 2). The phenotypic and biochemical characteristics of the isolate matched with LAB. 16S rRNA sequencing confirmed the isolate as *Pediococcus pentosaceus*. The strain *Pediococcus pentosaceus* DM101 obtained in this study is deposited in NCBI with accession number MK774704. The breast milk isolate obtained in this study fermented the carbohydrates: sucrose, galactose, maltose, raffinose, xylose, trehalose, melibiose, salicin, mannose, fructose and cellobiose without gas production, but failed to ferment lactose and arabinose. The results corroborate with the characteristics of *P. pentosaceus* CRAG3 that was isolated from fermented cucumber by Shukla and Goyal, (2014) [34]. Though isolated from milk, strain obtained in this study was incapable of fermenting lactose. Non-lactose fermenting strains of *P. pentosaceus* have been reported in fermented millet beverage (Oh and Jung, 2015) [23]. There are reports of isolation of lactose fermenting *P. pentosaceus* DMG01 from goat milk (Amrutha, 2019) [2] and other non-dairy sources (Semjonovs and Zikmanis, 2007) [31].

Table 1: Physiological Characteristics of *P. pentosaceus* DM101

Characterization	Result
Growth at	
pH 4.5	+++
pH 7.0	++++
pH 8.0	++++
pH 9.0	-
4°C	++
15°C	++
37°C	++++
45°C	+++
0% NaCl	+++
2% NaCl	+++
4% NaCl	+++
6.5% NaCl	++
10% NaCl	-

-No growth, + Very less growth, ++ less growth, +++ moderate growth, ++++ heavy growth

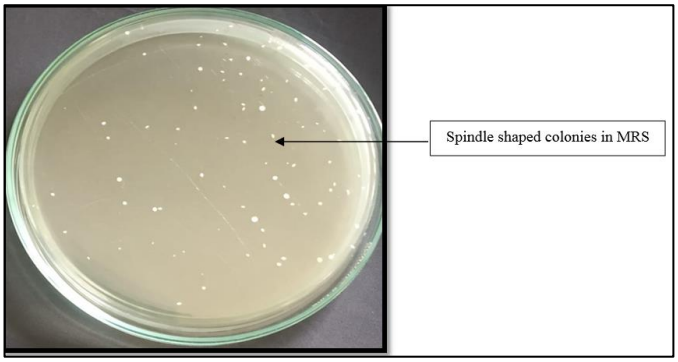

Fig 1: Colony characteristics of *P. pentosaceus* DM101 on MRS agar

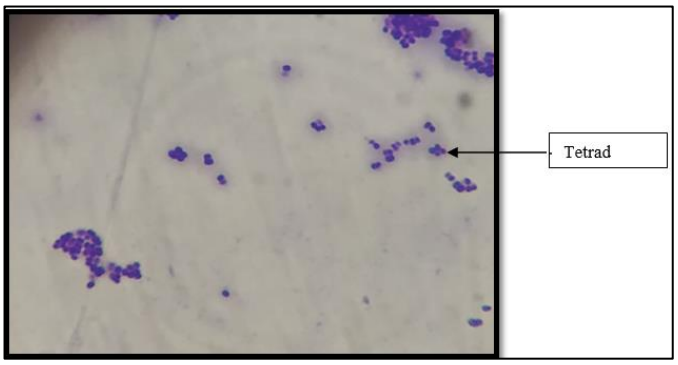

Fig 2: Microscopic examination of *P.pentosaceus* DM101

3.2 Probiotic properties of the isolate

3.2.1. Acid and Bile tolerance

Probiotic organisms should essentially survive the stress environments of acid and bile to reach their target site of action. Remarkable number of cells survived even after 4h of exposure to pH 3.0 (Fig 3). Kavitha and Devasena (2013)[14] also reported similar tolerance results. The inhibitory effect became very pronounced when the acidity shifted to pH 2.0. An interesting observation made in this study was that when exposure time increased to 3h, the isolate gradually acquired endurance to acid environment. A similar trend in the tolerance pattern has been reported for *P.pentosaceus* SW01 after 3h of exposure to pH 2.0 (Oh and Jung, 2015) [23]. Contrary to this, Osmanagoglu *et al.* (2010) [24] reported that to the breast milk isolate *P.pentosaceus* OZF could not withstand pH 2.0 even for 1h.

Gilliland *et al*. (1984) [11] proposed a bile concentration of to 0.3 per cent for screening bile resistant strains. The isolate in this study showed remarkable resistance to both 0.3and 0.6% bile salt concentration (Fig 4).

Similar tolerance to bile salts has been reported earlier for breast milk isolates (Diba *et al.,* 2012) [7]. Ozusagalam *et al.* (2018) [25] observed 100% survivability for breast milk isolates even after 4h of exposure to one percent bile salt. Observations made in this study underscore the remarkable acid and bile tolerance of *P. pentosaceus* DM101.

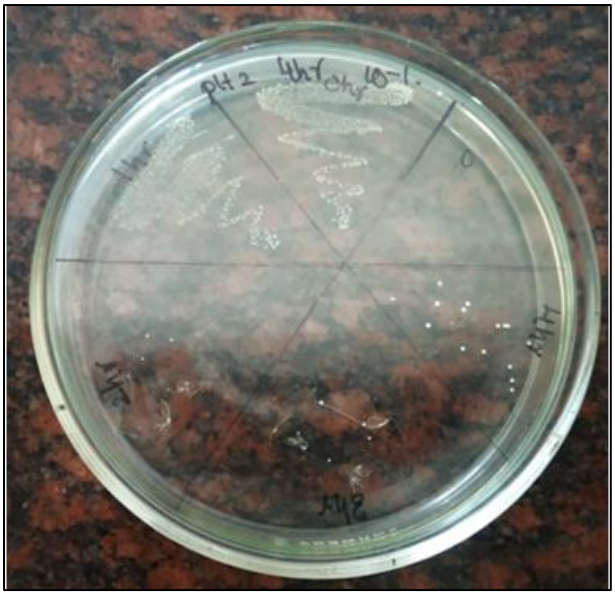

Fig 3: Acid tolerance of *P.pentosaceus* at pH 2.0

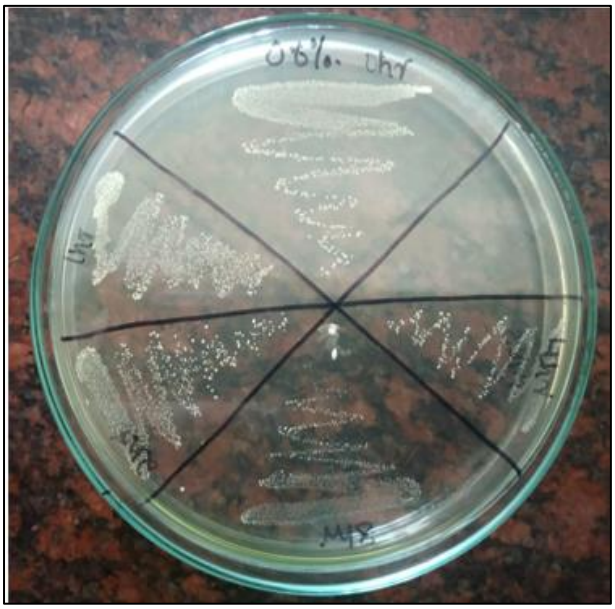

Fig 4: Bile tolerance of *P.pentosaceus* at 0.6% bile

3.2.2 Adhesion potential

Cell surface characteristics are critical for probiotic strains as they indicate their ability to adhere to mucosal surfaces in the gastrointestinal tract. The beneficial properties that could be impacted by the probiotics are very much related to the colonization of the host by probiotic bacteria. Autoaggregation permits the cells to persist in the intestinal mucosa and thereby promotes their beneficial effects. *P.pentosaceus* DM101 isolated in this work exhibited an autoaggregation of 68 percent. This value is in agreement with the reports of Lee *et al*. (2014) [18]. According to Zommiti *et al*. (2018) [40] strains with an autoaggregation of more than 40% can be graded as good and those with a value less than 10 percent as weak. Accordingly, breast milk isolate used in this study has good adhesion potential.

Cell-surface hydrophobicity (CSH) is reflective of the possible interactions the probiotic cell can have with the host intestinal mucosa. CSH value of 60 percent with apolar solvent xylene affirms the hydrophobic cell surface for the breast milk isolate. The hydrophobic potential is dependent on the age of the cell, its surface chemistry and components of growth medium (García-Cayuela *et al.,* 2014) [10]. Serrano-Niño *et al*. (2016) [32] opined that a CSH value above 50 percent indicates high hydrophobicity. The positive correlation of CSH value to adhesion potential has been reported by Wadstroum *et al*. (1987) [39].

3.2.3 Safety assessment of the isolate

Safety evaluation of probiotic strains is obligatory in order to ensure no untoward effects happen from their consumption. No clearing of the zone was evident on blood agar (Fig 5). Both haemolysis and gelatin liquefaction were absent for this isolate. The results are suggestive of possible absence of virulence factors. Non hemolytic and non- gelatin liquefying strains of *Pediococcus pentosaceus* has been reported from goat milk (Amrutha, 2019) [2] and human breast milk (Osmanagaoglu *et al.,* 2010) [24].

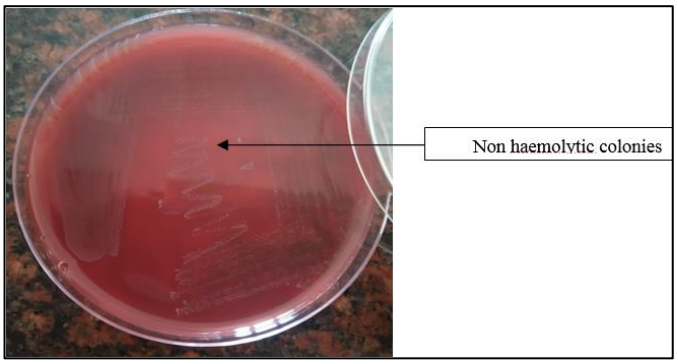

Fig 5: *P. pentosaceus* DM101 in blood agar plate

Pediococcus pentosaceus DM101 isolated from breast milk was found to be sensitive /intermediate sensitive to Erythromycin, Chloramphenicol, Tetracycline, Azithromycin, Linezolid, Cephalothin, Gentamycin, Penicillin G, Co-Trimoxazole, Amoxicillin, Enrofloxacin, Ofloxacin, Ciprofloxacin, Streptomycin. The isolate was resistant to Vancomycin. Intrinsic resistance of *P.pentosaceus* to Vancomycin has been reported in many other studies also (Osmanagaoglu *et al*., 2010)[24]. It was also resistant to cell wall synthesizing inhibitors like beta-lactam antibiotics Cephalosporins and Bacitracin (Sarkar *et al*., 2017) [30]. Parallel resistance between beta-lactam antibiotics and Cephalosporins was reported by Grimm, (1984) [12]. Varied response among Cephalosporins was observed by different strains *Pediococcus pentosaceus* (Singla *et al*., 2018)[35].The antibiogram profile shown by the isolate can be assumed to be reflective of the medication history of the feeding mother.

3.3 Functional properties of *Pediococcus pentosaceous* DM101

Kumar and Anad (1998) [16] reported a positive correlation

with EPS production and adhesion potential. The congo red assay of *Pediococcus pentosaceus* DM101 confirmed EPS production (Fig 6). However on repeated culturing, sliminess reduced. Black colored colonies were not present in Congo red agar when non-slimy colonies of the isolate were streaked. There are reports of loss in the ability to produce EPS by lactic acid bacteria due to frequent subculturing, prolonged periods of incubation or incubation at high temperatures (De Vuyst *et al*., 1999) [6]. Genes responsible for EPS is often located in plasmids rather than the chromosome (Donot *et al*., 2012) [8]. Being a plasmid located trait, EPS production potential can be easily lost. This is one of the main technological problems associated with the use of such cultures.

Fig 6: Colony characteristics of *P. pentosaceus* DM101 on Congo red agar

The IC 50 value of the isolate was found to be 20.78mg/ml. The scavenging activity of *P.pentosaceus* increased with an increase in the volume of the sample used. The scavenging effect was found to be as high as 91.86 percent when the sample volume was 1.25 ml. The antioxidant activity of *Pediococcus pentosaceus* AR243 as determined by DPPH assay was 10.32% (Lin *et al*., 2018) [19] and of *P. pentosaceus* DMG01,a goat milk isolate was 35.45%(Amrutha,2019)[2]. Differences in observation could be due to strain variation and differences in the experimental conditions. The role of membrane dipeptidases (Attri *et al.*, 2012) [3] and exopolysaccharides (Amrutha, 2019) [2] in imparting antioxidant activity have been reported. The observations made in this work indicates that *P.pentosaceus* DM101 is a good probiotic candidate to be used for dietary intervention

Conclusions

Pediococcus pentosaceus DM101 isolated from human breast milk was found to be a good probiotic candidate based on *in vitro* studies. This non-lactose fermenting isolate, with its good antioxidant potential and remarkable endurance to extreme environments has immense potential to be explored in designing non-dairy based functional foods. Incorporation of probiotics in foods will be a good approach to supply dietary antioxidants. Well controlled biological studies and in depth molecular level work is need to be carried out before claiming the benefits.

4. Acknowledgment

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