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Microbial analysis of lactic acid bacteria in homemade fermented milk products

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ABSTRACT

Background: Fermented milk has long been part of human diets, relying on natural fermentation by Indigenous microbes, especially lactic acid bacteria (LAB), which contribute to flavour, preservation, and probiotic benefits. However, the microbial makeup of homemade varieties, particularly from less-studied regions, is poorly understood. **Method:** The research looked at 120 samples of fermented milk prepared at home from five different rural regions in Babylon Province. The lactic acid bacteria (LAB) were detected by morphological, biochemical, and molecular techniques, such as 16S rRNA gene sequencing and phylogenetic analysis with MEGA X. Isolation of the bacteria was done using MRS agar. To further understand the patterns of resistance, antibiotic susceptibility testing was also performed. **Results:** LAB counts varied across regions, with the highest in Area C (7.9×10^8 CFU/mL). Out of 120 isolates, 46 were identified as Lactobacillus species, some with probiotic potential based on phylogenetic analysis. However, many strains showed multidrug resistance (MDR), particularly to β-lactam and macrolide antibiotics. **Conclusion:** This study reveals significant microbial diversity in homemade fermented milk, highlighting the importance of preserving traditional fermentation methods. It also underscores the need to monitor MDR lactic acid bacteria (LAB) due to their potential health risks and role in spreading antibiotic resistance

Introduction

Lactic acid bacteria from the gram-positive non-spore-forming microbes ferment carbohydrates to LAB as the primary metabolic end product [1-2]. Bacterial fermentation preserves the nutritional value of foods and enhances the shelf life of fermented milk products [3]. Furthermore, LAB aids in formulating flavour compounds, modifying texture, and producing bioactive peptides that have probiotic effects and aid in maintaining gut health [4]. The composition of LAB and the milk used, environmental factors, fermentation time, and the producer's techniques are also important.

In cultured buttermilk and yoghurt, Lactic acid fermentation and alcoholic fermentation occur. This was also commonplace in ancient China. As a part of ancient civilizations' diets, fermented milk products are beneficial for health and nutrition, and handling them with care serves optimal well-being

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[5]. Varieties of cultured buttermilk and yoghurt are significantly common among diverse people, both culturally and globally. Such food is generally made through artificial means by natural fermentation processes employing microorganisms from the surroundings from old batches. The fermentation process increases the shelf life, sustains the food value, and enhances the taste by preserving and improving the sensory qualities of dairy products [6-7].

Live bacteria or yeasts, known as probiotics, can improve host health when administered in adequate doses [8-9]. There has been a lot of buzz around probiotic foods recently, thanks to the rising tide of health consciousness. The genus Bifidobacterium and the species Lactobacillus are home to several probiotic strains. Probiotic bacteria include streptococcus, enterococcus, bacillus, propionibacterium, and saccharomyces yeast [10, 11].

Lactobacillus acidophilus has a long history of human consumption in fermented foods like dairy. Because of their importance in fermented foods and their ability to produce antimicrobial chemicals that promote probiotic traits, LABs are currently the focus of substantial international research [12]. These characteristics include, but are not limited to, antitumor activity, relief of lactose intolerance [13-14], a decrease of serum cholesterol [15], stabilization of gut microflora [16], and stimulation of the immune system [17]. The LAB strain that is often used to make exopolysaccharides is actually utilized to make fermented milk, which makes it thicker and smoother [18]. The health-promoting properties of mannitol are thought to be produced by some LAB strains [19].

Bacteria are the most numerous of the many microorganisms found in the gastrointestinal tract (GIT). The gastrointestinal tract is home to a wide variety of yeasts, molds, and archaeal domains [20]. It is believed that probiotic microbes offer several health advantages. In addition to improving nutritional status, they fight colon cancer, cholesterol, inflammation, and other environmental pathogens by acting as an antibacterial agent and stimulating the host's mucosal and systemic immune responses. Diarrhea, lactose intolerance, and allergic responses can all be alleviated by them as well [20]. The ability of multistrain or multispecies probiotic mixtures to provide additive or synergistic effects as well as a wider range of health benefits has led to their increasing popularity [21, 22].

Progress in molecular biology studies and high-throughput sequencing technologies allow more precise characterization of microbial communities in fermented foods. Such tools enable LAB species to be identified more precisely and their functional roles in complex ecosystems to be examined. So far, studies have demonstrated a rich diversity of LAB genera such as Lactobacillus, Streptococcus, Leuconostoc, and Lactococcus, which differ not only in their contribution to fermentation but also in the characteristics of the final product.

This study focuses on LAB's diversity, molecular characterization, and antibiotic resistance spectrum, particularly Lactobacillus spp., from homemade fermented milk collected from rural Babylon while evaluating their probiotic potential and safety.

This study's results can enhance understanding of the microbiota of traditionally fermented milk and provide molecular aspects of it. They address the problems of drug resistance and the safety of locally produced food and probiotics.

Materials and Methods

Samples

For this study, 120 dairy product samples were collected from five rural areas in Babylon Province, 22 from each area, where residents sell dairy products in the markets. Each sample was collected in an aseptic environment. Then, 90 ml of peptone was used to homogenize the samples. In the enrichment stage, 100 ml of Mann, Rogosa, and Sharpe (MRS) broth was mixed with 10 ml of the homogenized solution, and the mixture was incubated for 24 hours at 37°C in a flask filled with anaerobic carbon dioxide. To avoid yeast contamination, nystatin was added to the medium. One gram of raw material was dissolved in nine ml of phosphate-buffered saline (PBS) to contain heterotrophic bacteria. Subsequently, a ten-fold serial dilution was performed. A 100 µl sample was added to each plate after the last three dilutions, and the plates were then incubated for 24 hours at 37°C.

Ethical Approval

The College of Science at Thi-Qar University approved the study project, and this approval was obtained in accordance with their document issue 722 in 3/1/2025. Also, the permission was obtained from animal breeders before samples were taken, and they were informed of the risks of milk-borne bacteria, milk sterilization

methods, and how to avoid the risk of contamination.

Bacterial Isolation

A small amount (1 g or 1 mL) of the sample was first homogenized in 9 mL of sterile peptone water or phosphate-buffered saline. Serial dilutions (10^{-1} to 10^{-6}) were prepared, and 0.1 mL from appropriate dilutions were inoculated onto De Man, Rogosa, and Sharpe (MRS) agar, a selective medium for Lactobacillus spp. The plates had been incubated anaerobically at 37°C for 48–72 hours using an anaerobic jar or CO_2 incubator. Colonies appearing creamy, circular, and off-white had been selected and subcultured on fresh MRS agar to obtain pure isolates. Gram staining had been performed to confirm that the isolates were Gram-positive rods, and a catalase test had been done to ensure they were catalase-negative. Further biochemical or molecular identification had been conducted to confirm the species. Pure cultures had been stored on MRS slants at 4°C or in glycerol broth at –80°C for long-term use [23].

Antibiotic Susceptibility Test

Fresh bacterial cultures were suspended in sterile saline to achieve a turbidity equivalent to 0.5 McFarland standards and then evenly spread onto the surface of Mueller-Hinton agar supplemented with 5% defibrinated sheep blood to support the growth of fastidious LAB strains. Agar was treated with a sterile cork borer to form wells where 100 µL of various antibiotic discs were placed in clinical use. The plates were pre-diffused for 1 hour at room temperature to facilitate the diffusion of the antibiotics, followed by anaerobic incubation at 37°C for 24 hours. Following incubation, the inhibition zones were measured in mm and interpreted using CLSI guidelines for non-fastidious organisms [24]. This information is critical for determining the safety and usefulness of these Lactobacillus strains as probiotics in the food and health industries.

Molecular Analysis

Following the specified guidelines and making minor changes to improve cellular lysis like including an additional lysozyme (20 mg/mL) incubation step at 37°C for 30 minutes, Genomic DNA was extracted from pure cultures of isolated Lactobacillus species with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). For Quality control and verification of concentration, the extracted DNA was evaluated with a Thermo Fisher Scientific NanoDrop ND-1000 spectrophotometer and kept at -20°C until further analysis. For molecular characterization, the 16S rRNA gene was cloned via PCR employing universal bacterial primers LPW57: (5'-AGAGTTTGATCCTGGCTCAG-3') and Uni_1492R: (5'-TACGYTACCTTGTTACGACTT-3') based on previously published work by [25]. Each reaction comprised 25 µL including 12.5 µL of DreamTaq Green PCR Master Mix (Thermo Fisher Scientific), 1 µL of each primer (10 pmol), 2 µL of template DNA, and 8.5 µL of nuclease-free water.

The following conditions were used to conduct the amplification in a Veriti® Thermal Cycler (Applied Biosystems, USA): a three-minute initial denaturation at 95°C, thirty seconds of annealing at 55°C, ninety seconds of extension at 72°C, and finally, five minutes of final extension at 72°C. A 1.2% agarose gel stained with ethidium bromide was used for electrophoresis of the amplified products, which were then visible under ultraviolet light. Sanger sequencing was employed at Macrogen Inc. (Seoul, South Korea) to sequence the positive PCR results, which were purified using the QIAquick PCR Purification Kit (Qiagen). In order to accurately identify isolates at the species level, sequence readings were run via BLASTN against the NCBI database. A sequence similarity of ≥ 99% was deemed necessary for reliable identification. Isolates were confirmed to be members of the Lactobacillus genus using phylogenetic analysis performed using MEGA X software.

Results

Isolation and Enumeration of Lactic Acid Bacteria (LAB)

The analysis of LAB counts across five regions revealed significant variation, with mean values ranging from as low as 4.5×10^6 CFU/mL in Area B to as high as 7.9×10^8 CFU/mL in Area C, indicating that local environmental factors, traditional fermentation practices, hygiene conditions, and raw material quality play crucial roles in shaping microbial load. As for Area C, while exhibiting the narrowest range of LAB count compared to the other areas, it also shows the highest overall count. This may indicate consistent fermentation conditions or stable starter cultures beneficial for rapid acidification, food safety, and long-term preservation. In contrast, Areas B and E recorded the lowest LAB counts (4.5×10^6 and 5.2

× 10^6 CFU/mL, respectively), potentially due to poor handling practices, exposure to unfavourable temperatures, or the presence of inhibitory substances such as residual antibiotics in milk, all of which can slow fermentation and increase contamination risk. Additionally, the wide variability observed in some regions, particularly in Area B (ranging from 3.2 × 10^5 to 9.1 × 10^7 CFU/mL), underscores the unstandardized nature of homemade fermentation processes, which can affect product consistency and microbial quality.

Morphological and Biochemical Characterization

Of the 120 isolates, 46 were confirmed as Gram-positive, catalase-negative rods, consistent with typical LAB morphology. These isolates were further subjected to biochemical testing and molecular identification. Microscopic examination revealed rod-shaped cells in most isolates, indicating dominance of the genus Lactobacillus.

Antibiotic Susceptibility Profile

A total of 46 Lactobacillus spp. Isolates were subjected to antibiotic susceptibility testing against 12 commonly used antibiotics across different classes (Table 1). The findings revealed a varied pattern of resistance and sensitivity. High levels of resistance were observed against Ampicillin (93%), Erythromycin (87%), Penicillin G (78%), and Gentamicin (76%), indicating a concerning resistance trend toward β-lactam and macrolide antibiotics. Conversely, relatively higher sensitivity rates were seen with Levofloxacin (85%), followed by Trimethoprim/Sulfamethoxazole (67%) and Streptomycin (65%). Moderate resistance patterns were detected for Vancomycin, where 52% of isolates were sensitive and 48% resistant. Resistance to Clindamycin (63%), Chloramphenicol (65%), and Tetracycline (70%) also remained significant. Notably, only 7% of the isolates were sensitive to Ampicillin, the lowest sensitivity rate among all tested antibiotics.

Phylogenetic Analysis

Figure 2 presents a phylogenetic tree based on the 16S rRNA gene sequences of Lactobacillus isolates obtained from homemade fermented milk products collected. The tree was constructed using MEGA X software to illustrate the genetic relatedness between the isolated strains and reference strains in the NCBI database, thereby enabling precise species-level identification and genotype classification. Each isolate, labelled according to its sample origin or isolate code, clustered with known Lactobacillus species such as L. acidophilus, L. plantarum, L. rhamnosus, and others, depending on sequence similarity (≥99% identity by BLASTN analysis). Bootstrap values indicate the reliability of branching patterns, supporting the robustness of the phylogenetic groupings.

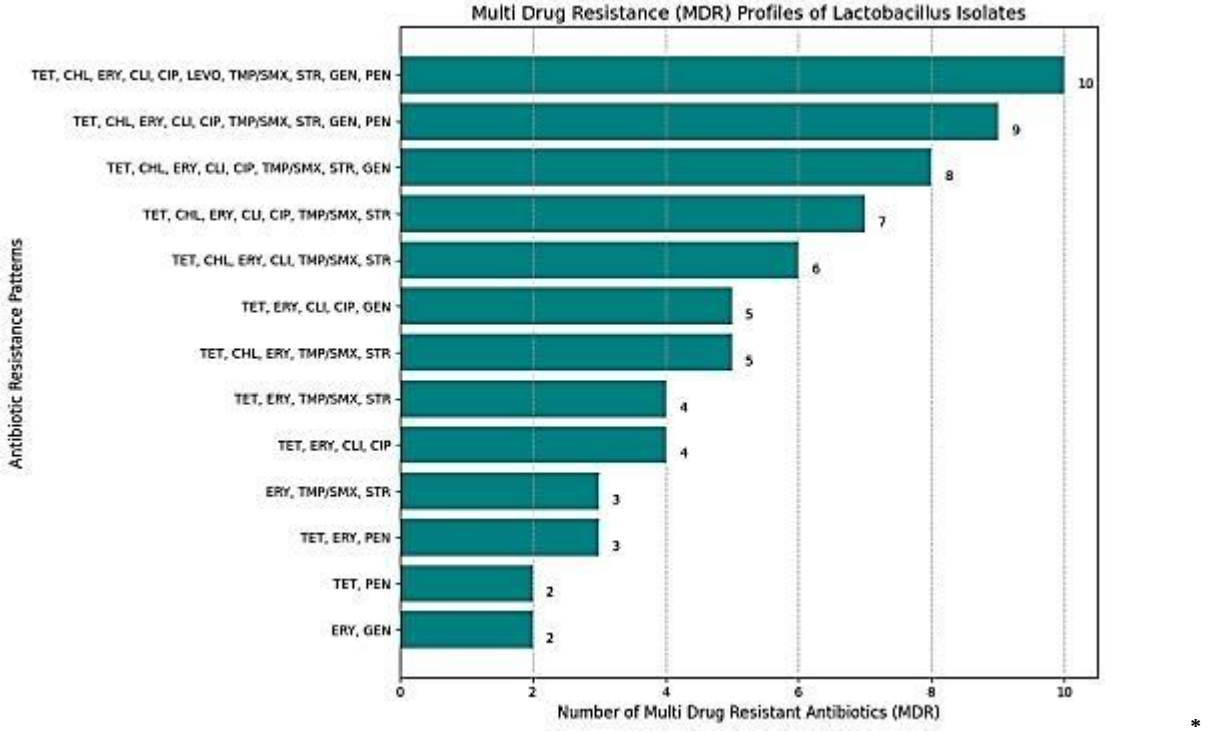
Table 1: Lactic Acid Bacteria (LAB) Counts Across Different Regions

Region	Sample size	Mean lab count (cfu/ml)	range of lab counts (cfu/ml)	Observations
Area A	22	6.3 × 10^7	5.1 × 10^6 – 7.8 × 10^8	Moderate
Area B	22	4.5 × 10^6	3.2 × 10^5 – 9.1 × 10^7	Low
Area C	22	7.9 × 10^8	6.7 × 10^7 – 8.2 × 10^8	Highest
Area D	22	3.4 × 10^7	2.1 × 10^6 – 5.6 × 10^7	Low
Area E	22	5.2 × 10^6	4.0 × 10^5 – 6.8 × 10^6	Lowest

Table 2: Antibiotic Susceptibility Profile of Lactobacillus spp. Isolates (n = 46)

Antibiotic	Sensitive (%)	Resistant (%)
Tetracycline	14 (30%)	32 (70%)
Chloramphenicol	16 (35%)	30 (65%)
Erythromycin	6 (13%)	40 (87%)
Clindamycin	17 (37%)	29 (63%)
Ciprofloxacin	20 (43%)	26 (57%)
Levofloxacin	39 (85%)	7 (15%)
Trimethoprim/ Sulfamethoxazole	31 (67%)	15(33%)
Streptomycin	30 (65%)	16 (35%)
Gentamicin	11 (24%)	25 (76%)
Penicillin G	10 (22%)	36 (78%)
Ampicillin	3 (7%)	43 (93%)
Vancomycin	24 (52%)	22 (48%)

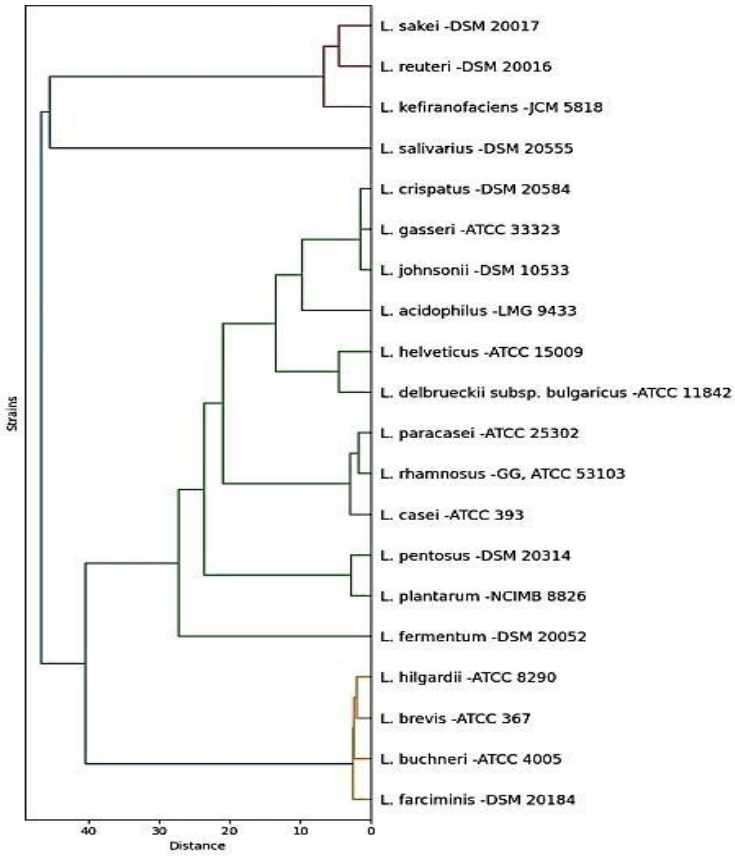
Figure 1: Assessment of Antibiotic Susceptibility and Emergence of Multidrug Resistance (MDR) Among *Lactobacillus spp.* in Homemade Fermented Milks



*

(TET)Tetracycline, (CHL) Chloramphenicol, (ERY) Erythromycin, (CLI) Clindamycin, (CIP) Ciprofloxacin, (LEVO), Levofloxacin, (TMP/SMX), Trimethoprim/Sulfamethoxazole, (STR) Streptomycin, (GEN), Gentamicin, (PEN) Penicillin G.

Figure 2: Phylogenetic analysis and molecular identification of the isolated strains



Discussion

Our isolation and enumeration data showed significant variation in LAB counts across the five sampled areas, with Area C exhibiting the highest mean count (7.9×10^8 CFU/mL), while Areas B and E had notably lower counts. This variability is consistent with findings from studies conducted in rural parts of Africa and Asia, where differences in hygiene practices, ambient temperature, and raw milk quality were shown to influence microbial density in artisanal dairy products. For example, a study by [26] on traditional fermented milk from Nepal reported similar fluctuations in LAB populations depending on local production conditions. Notably, stable starter cultures, or optimal fermentation conditions as evidenced by elevated LAB levels, appear to be a characteristic of Area C, which may have socioculturally been influenced by knowledge transfer across generations, underscoring the significance of indigenous fermentation practice.

Morphological and biochemical characterization confirmed 46 out of 120 isolates as Gram-positive, catalase-negative rods characteristic of Lactobacillus species. Such a low confirmation rate could suggest the presence of other genera of LAB or non-LAB contaminants, highlighting the need for selective isolation methods and additional molecular techniques for definitive identification. Most confirmed isolates showed rod-shaped cells microscopically, which aligns with the expected morphology of Lactobacillus strains. These findings corroborate earlier work, including that of [27], where greater proportions of lactobacilli were reported due to the use of selective media and standardized culture conditions, supporting the assertion that lactobacilli dominate traditional fermented dairy products. The other bacterial contaminants we observed may be attributed to the openly spontaneous fermentation nature of homemade systems, which enhances microbial diversity while posing risks of contamination or spoilage from pathogenic microorganisms.

Testing for antibiotic susceptibility showed troubling resistance levels with the Lactobacillus isolates, especially concerning the β-lactam and macrolide antibiotics. There was a high resistance rate towards Ampicillin (93%), Erythromycin (87%), Penicillin G (78%) and Gentamicin (76%). This has shown an emerging strain of MDR bacteria in these traditional fermentation products. This widespread resistance may be linked to prior exposure to antibiotics used in livestock farming, contamination of raw materials, or the horizontal transfer of resistance genes within microbial communities. Of particular concern is the high resistance to Erythromycin and Ampicillin, which are commonly prescribed in clinical settings, raising public health concerns regarding the potential transmission of resistant strains through food chains. Conversely, higher sensitivity was observed to Levofloxacin (85%), Trimethoprim/Sulfamethoxazole (67%), and Streptomycin (65%), suggesting that some antibiotics remain effective against these isolates. Vancomycin showed intermediate resistance, with nearly equal distribution between sensitive and resistant isolates, indicating variable responses within the population.

A comparative analysis with a European Food Safety Authority (EFSA)-approved Lactobacillus strain database indicates that most commercially used strains exhibit much lower resistance to these clinically important antibiotics. For instance, studies by [28] and [29] found that industrial Lactobacillus strains generally retain high sensitivity to β-lactams and macrolides, making them safer candidates for probiotic applications. In contrast, our findings suggest that some locally isolated strains may have acquired resistance genes through environmental exposure, possibly via antibiotic residues in raw milk or horizontal gene transfer within complex microbial communities. This raises concerns regarding their use in food-grade applications without prior safety screening.

These resistance patterns highlight the need for careful evaluation of LAB isolates before their application in food or probiotic industries. While Lactobacillus species are generally considered safe (GRAS) and widely used in food fermentations, antibiotic resistance determinants pose a potential risk for gene transfer to pathogenic bacteria. Therefore, regulatory guidelines recommend screening LAB for antibiotic resistance as part of safety assessments. The observed MDR profiles in this study emphasize the importance of monitoring antimicrobial resistance in non-clinical settings, especially in homemade food systems where microbial dynamics are less controlled.

With the aid of 16S rRNA gene sequences, the phylogenetic analysis confirms that the remains of Lactobacillus species, including L. acidophilus, L. plantarum and L. rhamnosus, were well recognized. These species are well known for their probiotic properties, modulation of immune functions, and the ability to exhibit antagonistic activities against pathogenic microorganisms. The coexistence of MDR with these probiotic strains poses a paradox because these strains can be tailored for their beneficial attributes, but also caution must

be exercised so that they do not act as reservoirs of resistance genes. The literature on Lactobacillus shows that, for some reason, non-commercially sourced isolates tend to have more MDR than commercially sourced isolates, particularly in developing countries with lax regulations on agricultural antibiotic use. In South Africa, A study [30] conducted conducted which found traditionally fermented milk had similar resistance patterns and reinforced the theory that unregulated antibiotic-laden livestock feed is driving the development of resistant LAB strains.

Conclusion

This study successfully characterized Lactobacillus strains' diversity and antibiotic resistance profiles sourced from homemade fermented milk. This highlighted the strain's potential as probiotics whilst concerning the prevalence of MDR bacteria. The molecular identification and phylogenetic analysis revealed important aspects of the microbial population in traditional fermentation processes within the scope of evolutionary biology. Further work should investigate these strains' purported probiotic effectiveness, some of which may include their viability under gastrointestinal conditions and associated health advantages, including anti-inflammatory effects. More importantly, studies of antimicrobial resistance transfer mechanisms between LAB and studies designed to monitor resistance trends over time would be important. Standardized fermentation and defined hygiene practices would limit the spread of resistance, enabling safer application of probiotics whilst preserving traditional foods.

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