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Detection of lytic phage infecting flavour-producing strain of *Lacticaseibacillus paracasei* in the dairy effluents of Kerala

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Research Article

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Abstract

The performance of the starter culture is a critical factor that decides the quality of fermented milk. Dahi is a fermented milk product popular in India made using a mixed starter culture of lactic acid bacteria comprising acid and flavour producers. The prevalence of bacteriophages in the dairy environment can critically affect the activity of these starter cultures resulting in starter failure. As there is little information available on the occurrence of bacteriophages in the dairy environment of Kerala, this research communication examines the presence of lytic bacteriophages acting against three potential flavour-producing strains of *Lacticaseibacillus paracasei* (*Lc. paracasei*). Dairy effluent samples were screened for the presence of phages against the strains of *Lc. paracasei* by the multiple host enrichment method. Plates showing clearance zone in spot assay were confirmed for the presence of phages by double-layer agar assay. The plaques obtained in the double-layer agar assay were purified for further identification by next-generation sequencing. A bacteriophage infecting one of the three strains of *Lc. paracasei* was detected by the plaque assay and the blast annotation of the bacteriophage sequence found 86.05% similarity of the phage to *Siphoviridae* family. The study endorses the need for monitoring phages in the dairy environment to control phage-related starter failure in the state of Kerala.

Lc. paracasei is a homofermentative lactic acid bacterium that is commonly used as an adjunct starter in dairy fermentations. This species contributes to flavour development and is a potential probiotic candidate in fermented dairy products (Mercanti *et al*., 2016). The development of a robust starter culture for industrial application is a rigorous process involving the characterization of its technological and functional attributes along with an assessment of its resilience to various threats in the nonsterile environment. Dairy effluents are potent sources of virulent phages that can infect starter cultures. The presence of phage-sensitive bacteria in starters could trigger phage multiplication and produce lytic phages to a significant level, irrespective of their initial low concentration. Such phage attack delays or even puts a complete halt on fermentation, leading to inferior quality products. Bacteriophages attacking lactic acid bacteria are gaining attention as the knowledge of the host phage interaction is essential for effectively tackling the problems of starter failure (Sunthornthummas *et al*., 2017). This study aimed to screen dairy effluents for the presence of lytic phages infecting the potential flavour-producing strains of *Lc paracasei*.

Materials and methods

Bacterial culture and cultivation media

Three indigenous *Lc. paracasei* strains isolated from household dahi samples of Kerala were used for the study. The details of strains (online Supplementary Table S1) and media for propagation of host and bacteriophage are given in the Supplementary File.

Identification of Lc. paracasei *strains*

Lc. paracasei strains were identified using 16S rRNA gene sequencing. The 16S rRNA gene was amplified using the universal primers, namely forward primer 27f (5′ AGAGTTTGA TCCTGGCTCAG3′) and reverse primer 1492r (5′ GGTTACCTTGTTACGACTT 3′). The PCR conditions and sequencing details are given in the online Supplementary File. The sequences were compared to the GenBank database using the Basic Local Alignment Search Tool (BLAST).

RAPD analysis of *Lc. paracasei* strains was performed using random primer OPA-11, Operon Technologies, Alameda, CA (CAATCGCCGT). The phylogenetic tree was constructed using GelJ Version.2 Software. Pearson correlation was used as a similarity method and linkage was carried out with UPGMA with a tolerance of 10%.

Phage isolation and enrichment

Dairy effluent samples from a local milk processing unit were collected in sterile bottles and transported at 4°C to the laboratory for analysis. 50 ml of pooled sample was centrifuged at 4000 ***g*** for 10 min at 4°C. The supernatant was filtered through a 0.45 µm pore size mixed cellulose esters membrane syringe filter (Merck Millipore Ltd., Cork, Ireland) and stored at 4°C for further analysis (Feyereisen *et al*., 2019). The filtrate was then screened for the presence of bacteriophages by multiple host enrichment method with modification for *Lc. paracasei* host whose details are given in the Supplementary File (and see Vaiyapuri *et al.* 2021).

Phage detection and enumeration

Spot assay was used as the preliminary screening test for the detection of phages. Enumeration of phages was done by plaque assay using the double-layer agar plate method (Feyereisen *et al*., 2019).

Phage DNA isolation, library construction and sequencing

Phage DNA was extracted from the filtered stock lysates according to a standard phenol/chloroform method (Moineau *et al*., 1994). The sequencing of the phage genome was done at OmicGen Lifesciences Pvt Ltd, Ernakulam, India. Sequencing libraries were constructed using the NEB Ultra DNA kit (NEB, USA) according to the manufacturer's instructions for library preparation. The qualified library was used for sequencing. The library normalization, pooling and sequencing were done as per Kot *et al.* 2014. The genomes were sequenced as a part of a flow cell on the Illumina MiSeq platform and the pre-processed reads were *de novo* assembled using SPAdes (v3.13.1).

Genomic data availability

Assembled and annotated sequence of *Lacticaseibacillus paracasei* phage was submitted to www.ncbi.nlm.nih.gov GenBank under accession number MZ672003. The accession numbers of *Lc paracasei* strains are given in online Supplementary Table S1.

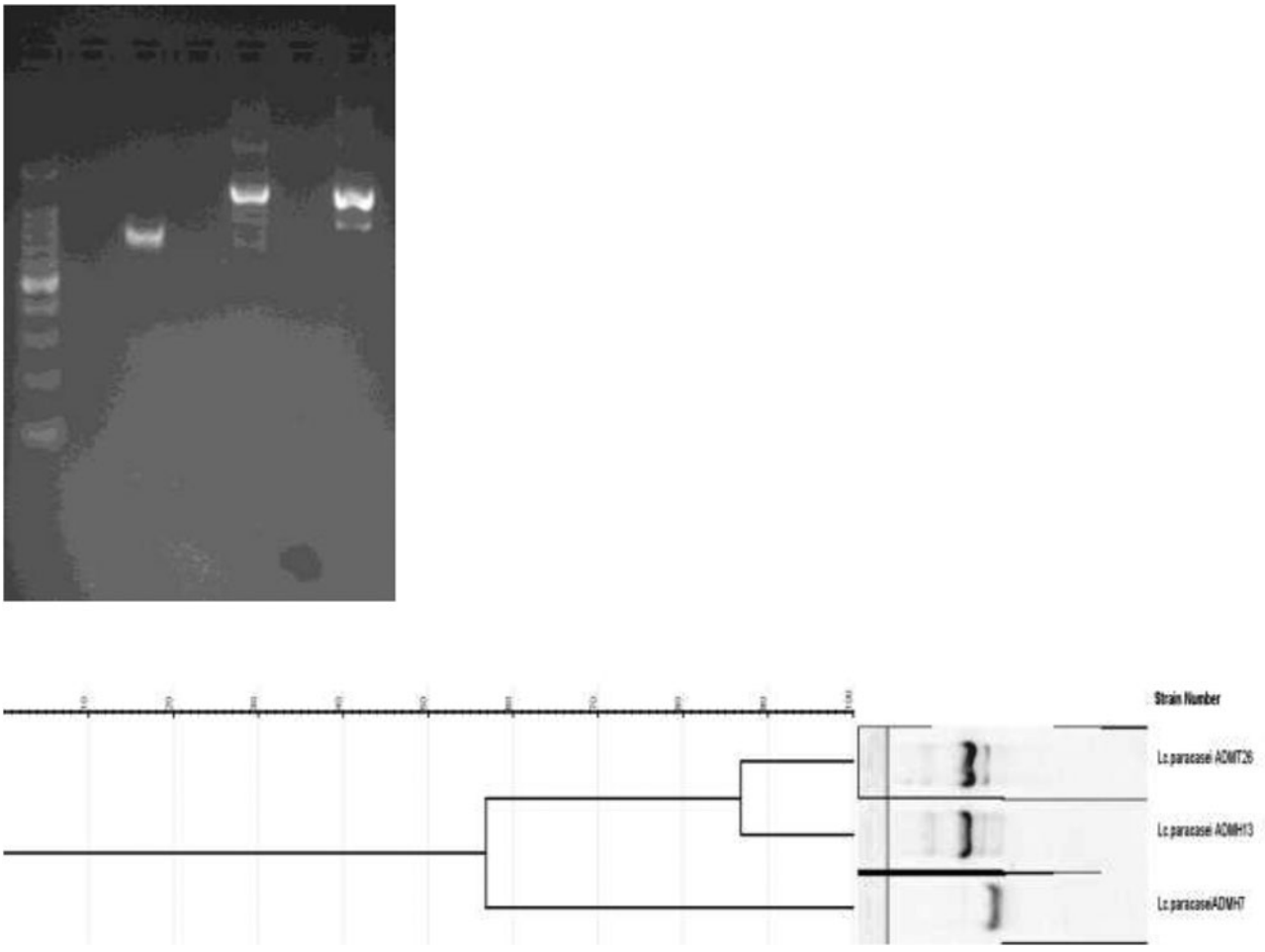

Figure 1. RAPD patterns and dendrogram of *Lc. paracasei* strains using the random Primer OPA-11. Lane-1 Ladder-100 bp; Lane 3- *Lc. paracasei* ADMH7; Lane 5- *Lc. paracasei* ADMH13; Lane 7 *Lc. paracasei* ADMT26.

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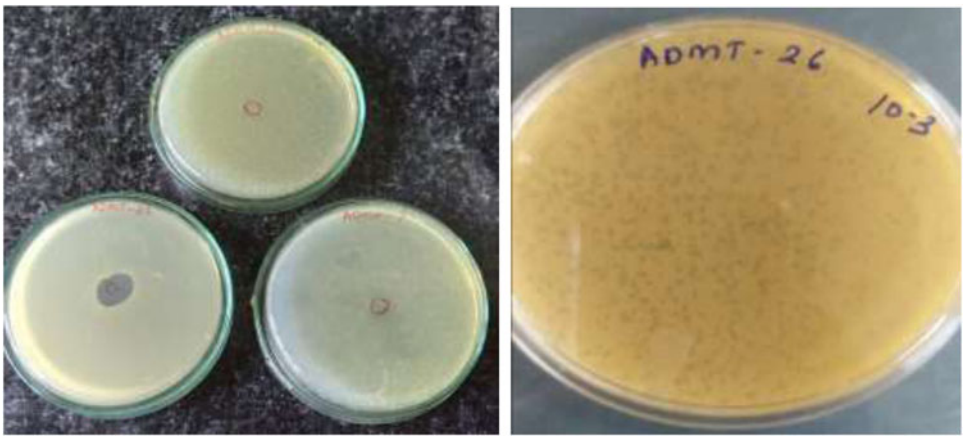

Figure 2. Plaques of Lphi2ADMT26 phage on *Lc. paracasei* ADMT26 lawn. (a) A clear zone of lysis was observed for ADMT 26 on spot assay. No zones were observed for ADMH 13 and ADMH 7 (b) Clear plaques were observed after double-layer agar plating of enriched phages.

Table 1. The fastq quality information of the phage Lphi2ADMT26

Sample	Raw reads	Raw data (G)	Clean reads	Clean data (M)	Q30 (%)	GC (%)
Lphi2ADMT26	15316517	2.29	5579307	18.375	0.96	58

Results and discussion

Based on the 16S rRNA gene sequences of length 1465 bp, the strains were identified as *Lc.paracasei*. The assembled sequences were deposited at NCBI GenBank database with accession number as shown in online Supplementary Table S1. RAPD analysis using the random primer OPA-11 revealed *Lc.paracasei* ADMH7 is significantly different from the other two strains and the other two are dissimilar at 10% level. The dendrogram and the RAPD patterns are given in Figure 1. After the enrichment of the effluent sample in the presence of three *Lc. paracasei* strains only one strain (*Lc. paracasei* ADMT 26) was found to be sensitive to the phage present in the effluent (Fig. 2a). The other two strains *Lc. paracasei* ADMH7 and *Lc. paracasei* ADMH13 were found to be resistant. The genetic variation of these strains revealed during RAPD analysis supports the difference in phage sensitivity. This phage lysing the strain *Lc. paracasei* ADMT 26 was designated as phi2ADMT26 and produced plaques with diameters 1–2 mm Figure 2b. After serial dilution, an average plaque count of 1×10^6PFU/ml was obtained. The fastq quality information of the phage Lphi2ADMT26 is given in Table 1. The largest contig, having a length of 3781 bp, was extracted and BLAST annotated. The blast annotation showed 86.05% identity with complete genomic sequence of the temperate bacteriophage (Accession number NC_048680) isolated from *Lactobacillus casei*, designated as *Lactobacillus phage LJ* belonging to *Siphoviridae* family with 2e-81 E-value and 7% query coverage according to the nucleotide homology. Assembled and annotated genome of *Lacticaseibacillus paracasei* phage was submitted to GenBank under accession number MZ672003.

Bacteriophages are the biological entities that are present in the ecosystems where bacteria are present, including man-made ecosystems like dairy fermentation vats and dairy effluents (Marcó *et al.*, 2012). *Lc. paracasei* is one of the species that is usually isolated from artisanal fermented milk and has been found to have good technological properties like flavour production and, potentially, health-promoting effects (Bengoa *et al.*, 2021). Many of the small-scale dairy units that venture into fermented milk product manufacturing in India are unaware of the consequences of phage attacks and are not sufficiently concerned about the proper disposal of effluent. *Lacticaseibacillus* phage Lphi2ADMT26 in this study is the first *Lc. paracasei* bacteriophage isolated from dairy effluents in India. The presence of *Lb. paracasei* phage ΦT25 was detected in abnormal fermented milk formed in a fermented milk factory in Thailand, and also belonged to the *Siphoviridae* family (Sunthornthummas *et al.*, 2017). The presence of lytic phages affecting technologically relevant strains can limit their application in the food industry. The commonly used method in India for tackling phage-related problems in dairy fermentation is starter rotation together with sanitation by chemical and thermal treatments. Adoption of starter rotation without the proper knowledge of phage presence in the environment can ultimately create an ideal environment for the build-up of phages. The virulent nature of phages along with their increased resistance to thermal and chemical treatments necessitates early detection and continuous monitoring. (Mahony *et al.*, 2012). The result of the present study highlights bacteriophage screening of starter culture collection as a good choice for use in industrial dairy fermentation to get consistent quality products.

In conclusion, we have characterized bacteriophage sensitivity of the dairy starter culture species, *Lc. paracasei*. The phage-resistant strains that we identified can be used effectively as functional starters in the manufacture of fermented milk products. As the study of phages affecting potential starters like *Lc. paracasei* is still in its infancy in India, screening of phage samples from other niches in dairy premises is also necessary to explore their mode of action and to devise evolutionary strategies.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029923000274.

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