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ORIGINAL ARTICLE

Molecular characterization of lactic acid bacteria recovered from natural fermentation of beet root and carrot Kanji

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Abstract The lactic acid bacteria (LAB) play an important role in the fermentation of vegetables to improve nutritive value, palatability, acceptability, microbial quality and shelf life of the fermented produce. The LAB associated with beetroot and carrot fermentation were identified and characterized using different molecular tools. Amplified ribosomal DNA restriction analysis (ARDRA) provided similar DNA profile for the 16 LAB strains isolated from beetroot and carrot fermentation while repetitive extragenic palindromic PCR (rep-PCR) genotyping could differentiate the LAB strains into eight genotypes. Thirteen strains represented by five genotypes could be clustered in five distinct groups while three LAB strains exhibiting distinct genotypes remained ungrouped. These genotypes could be identified to be belonging to *L. plantarum* group by 16S rDNA sequencing. The *recA*-nested multiplex PCR employing species-specific primers for the *L. plantarum* group members identified the LAB strains of six genotypes to be *L. paraplantarum* and the other two genotypes to be *L. pentosus*. Three genotypes of *L. paraplantarum* were consistently found on the third and sixth day of beetroot fermentation whereas a distinct genotype of *L. paraplantarum* and *L. pentosus* appeared predominant on the tenth day. From carrot Kanji two distinct genotypes of *L. paraplantarum* and one genotype of *L. pentosus* were identified. REP-PCR DNA fingerprinting coupled with 16S rDNA sequencing and *recA*-nested multiplex PCR could clearly identify as well as differentiate the diverse *L. plantarum* group strains involved in the fermentation.

Keywords Identification · Lactic acid bacteria · Kanji · REP-PCR · *recA*-multiplex PCR

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Introduction

Intake of vegetables rich in antioxidants and other nutritional components is suggested to provide various health benefits to humans. The lactic acid fermentation of these vegetables, applied as a preservation method for the production of finished and half-finished products, is considered as an important technology because of its capability to improve the nutritive value, palatability, acceptability, microbial quality and shelf life of the fermented produce. In North India deep purple colored carrot is fermented along with crushed mustard seed, hot chili powder and salt for 7–10 days to get a popular drink called Kanji, which is considered to have high nutritional value and cooling and soothing properties [1]. A similar drink was made from beetroot [2], which is considered to have the potential to prevent infectious and malignant disease. In spite of being a popular drink in North India, very limited information is available on the lactic acid bacteria (LAB) associated with Kanji fermentation.

A correct identification and classification of otherwise diverse LAB involved in the natural fermentation is difficult without the support of genotypic techniques, which has been witnessed, in the case of closely related *Lactobacillus acidophilus* complex, *Lactobacillus casei* complex and *Lactobacillus plantarum* complex [3, 4]. Besides facilitating in identifying the bacterial species, it is expected that the

genotypic techniques used should also have the potential to reveal the strain peculiarities to differentiate the strains within a species, as the suitability/relevance of a species for any particular application is strain dependent. Partial or complete sequencing of 16S rDNA of the genotypes would lead to the identification of the bacterial species involved in the fermentation.

In recent years several molecular techniques like ribotyping [5], pulse field gel electrophoresis [6], multilocus sequence typing [7] have been used for the typing of bacterial strains. Random amplified polymorphic DNA (RAPD), which involves DNA amplification using randomly designed primers, has the potential to generate polymorphic DNA patterns but often exhibit poor reproducibility [8, 9] and may not be equally polymorphic when diverse microflora are analyzed. Alternatively, polymerase chain reaction (PCR) amplification of repetitive bacterial DNA elements (rep-PCR) has been recognized as a simple PCR-based technique with high discriminatory power, amenable for high thorough put analysis and capable of typing wide range of gram-negative and several gram-positive bacteria [10]. Among the various repetitive bacterial DNA elements, Repetitive Extragenic Palindrome proposed by Versalovic et al. [11] has been widely used for the molecular typing/grouping of wide range of microorganisms.

In this study, we intended to discriminate and identify LAB strains isolated from natural fermentation of beetroot using molecular methods based on DNA amplification. Genomic fingerprinting using amplified ribosomal DNA restriction analysis (ARDRA) on the one hand, and rep-PCR on the other, were used to study the species and strain level variations respectively among the LAB strains. The broad group of the LAB genotypes were then identified by partial 16S rDNA sequencing followed by identification of the correct species using the species specific PCRs. The rep-PCR genotypes identified among the LAB species/strains isolated during the course of beetroot fermentation were compared with that of representative LAB strains isolated from carrot fermentation carried out following the same procedure.

Materials and methods

Isolation and of lactic acid bacteria

Peeled/ grated/crushed beetroot (33%), salt (2%), coarsely ground mustard seeds (1.5%) and chili powder (0.015) were mixed in potable water and allowed to ferment in a long necked mud pot for 6–9 days. Three batches of fermentation were set and pooled samples drawn from these batches were used for isolating the LAB strains. de Man, Rogasa and Sharpe (MRS) agar (Hi-Media, Mumbai, India) plates containing 6% NaCl was used for the isolation of LAB in this study. To distinguish lactic acid producing bacteria from other bacteria, 1% of $CaCO_3$ was added to the MRS agar plates. Fermented beetroot samples drawn on 3rd, 6th and 10th days were appropriately diluted and spread on to the surface of MRS agar plates containing 6% NaCl and 1% $CaCO_3$. Plates were incubated under anaerobic conditions at 37°C for 2 days. Colonies of acid producing bacteria, identified by a clear zone around each colony, were randomly selected from the MRS plates and purified by replating on MRS agar plates. Colonies were reselected and initially Gram-stained and tested for the production of catalase. The isolates were further tested for their homo/hetero fermentative nature and their ability to ferment glycerol, D-xylose and melezitose.

PCR-RFLP of 16S rDNA

The total genomic DNA from the LAB strains was isolated following the method previously described [12]. Primers fd2 and rp1 described by Weisburg et al. [13] was used to amplify the 16S rRNA gene from the LAB strains. PCR was performed in a reaction volume of 50 µl with 0.5 µM each forward and backward primers, 5.0 µl of 10X PCR buffer, 1 mM of $MgCl_2$ (final conc), 100 µM each of dATP, dCTP, dTTP, dGTP and 1.5 U of *Taq* polymerase using the following program on an Eppendorf Master cycler (Germany): an initial denaturation (95°C for 3 min), followed by 30 cycles of denaturation (94°C for 30 sec), annealing (54°C for 1 min) and extension (72°C for 1 min). A final extension of 72°C for 6 min was given at the end of every PCR reaction. The PCR product obtained was visualized in 0.8% agarose gel and those with sufficient amplification were subjected to restriction analysis with two different restriction enzymes *Hae III*, and *Msp*1 (MBI Fermentas, USA). The restriction reactions were performed in a final volume of 15 µl with 8 µl of amplified product, 2 U of the corresponding restriction enzyme and its recommended buffer and incubated at 37°C for about 5 h. The digested products were run in a 1.5% agarose gel, stained with ethidium bromide and documented using Syn gene gel documentation system (USA).

DNA fingerprinting by rep-PCR

Rep-PCR fingerprinting was carried out using the REP1R-D (5′-IIIMCGBCGNCATCSGGC-3′) and REP2D (5′-MCGBCTTATCSGGCCTAC-3′) primers. Reaction mixtures for PCR amplifications contained the final concentrations of the following reagents (MBI Fermentas): 1× PCR buffer without $MgCl_2$; 3 mM $MgCl_2$; 200 µM each

of dATP, dCTP, dGTP, dTTP; 1.5 U of *Taq* polymerase; 10 ng of template DNA; 0.4 μM each primer and sterile milliQ water to a final volume of 20 μl. Reaction mixtures were raised to an initial temperature of 94°C for 5 min to denature the DNA. Thereafter, reaction mixtures were cycled for 30 sec at 94°C, 1 min at 50°C, and 2 min at 72°C for a total of 35 cycles, followed by a final polymerase extension step at 72°C for 8 min. A negative control (no template DNA) and a positive control (template DNA giving amplified product) were included in every PCR reaction. The primers used were custom synthesized from Qiagen-Operon (Germany GmBH). The amplified product was run in a 1.2% agarose gel along with 1 kb DNA ladder, at a constant voltage, and documented using Syn gene gel documentation system. The rep-PCR bands were analyzed and a dendrogram was constructed by unweighted pair group method with arithmetic mean (UPGMA) clustering method of TREECON software package.

Molecular identification of LAB

The 16S rDNA amplicons of four LAB strains representing different REP-PCR genotypes were purified by Qiaquick PCR cleanup kit (Qiagen, Germany) and custom sequenced (MWG Biotech, Bangalore, India). Sequence similarity searches were performed against the nucleotide sequence databases using the basic local alignment search tool (BLAST) program. To obtain a more detailed identification, even at subspecies level, the *recA*-nested multiplex-PCR assay was performed as described by Torriani et al. [14]. This multiplex-PCR assay uses a reverse primer (pRev) in combination with three species-specific forward primers (planF, pentF and paraF) to distinguish the closely related species of the *L. plantarum* group. The primers used were synthesized from MWG Biotech and the PCR reactions were performed in an Eppendorf Master cycler using the MBI Fermentas reagents.

Cloning and sequencing of the recA PCR amplicons

The *recA* PCR amplicons from the LAB strains K8, K9, K11, K13 were purified (Qiaquick PCR cleanup kit, Qiagen) and cloned into the linear plasmid vector pDRIVE supplied in the QIAGEN PCR cloning kit (Germany, GmbH), according to the manufacturer's protocol. Cloned fragments were custom sequenced using M13 forward primer. The identities of the *recA* sequence obtained from the LAB strains were verified by a national centre for biotechnology information (NCBI) megablast search against the nucleotide databases.

Nucleotide sequence accession numbers

The Gen bank accession numbers assigned to partial 16S rDNA sequences are EU 380190 (*L. pentosus* K8), EU 380191 (*L. paraplantarum* K9), EU 380192 (*L. paraplantarum* K11), EU 380193 (*L. paraplantarum K13*).

Results

A total of 16 bacterial strains isolated from beetroot and carrot fermentation were considered as belonging to the genus *Lactobacillus* based on their morphology, Gram-positive staining, absence of catalase activity and motility and ability to produce acid during growth. All the LAB strains used in this study were heterofermentative. The strains K2, K6, K8 could ferment glycerol and D-xylose but not melezitose. However, other LAB strains could ferment melezitose but not glycerol and D-xylose. They were further characterized by molecular methods. PCR amplification of 16S rDNA revealed a single band of 1,500 bp, which upon restriction analysis (ARDRA) revealed four different fragments with respect to the enzymes *Hae*III and *Msp*I. All the lactic acid bacterial strains exhibited similar ARDRA profiles with the enzymes used. REP-PCR fingerprinting produced approximately 12 different DNA fragments ranging from 250 bp to 2.8 kb (Fig. 1) that could be represented by eight REP-PCR genotypes (Table 1). Thirteen LAB strains clustered to form five distinct groups while three LAB strains K2, K4 and K7 remained unclustered. BLAST search analyses using the partial 16S rDNA sequence of the LAB strains K9 (612 bp), K13 (782 bp), K8 (544 bp) and K11 (253 bp) resulted in identifications above the 99% level. The LAB strains K9 and K11 shared 99–100% and 99% similarity with *L. plantarum* and *L. pentosus*, respectively; K8 shared 99% similarity with both *L. plantarum* and *L. pentosus* whereas K13 shared 99–100% similarity with both *L. plantarum* and *L. pentosus*.

The *recA*-nested multiplex PCR employing species-specific primers for the detection of *L. plantarum* (318 bp), *L. paraplantarum* (107 bp) and *L. pentosus* (218 bp) revealed 107 bp and 218 bp amplicons for 13 and three LAB strains, respectively (Fig. 3). Consequently 11 LAB strains of four REP-PCR groups and the ungrouped LAB strains K4 and K7 were identified to be *L. paraplantarum* while two LAB strains represented by one REP-PCR group and the LAB strain K2 were identified to be *L. pentosus*. BLAST search of nucleotide sequences from the cloned *recA* amplicons confirmed the identity of the PCR products amplified during the *recA*-nested multiplex PCR assays.

Three *L. paraplantarum* genotypes could be identified among strains recovered on the third and sixth day of

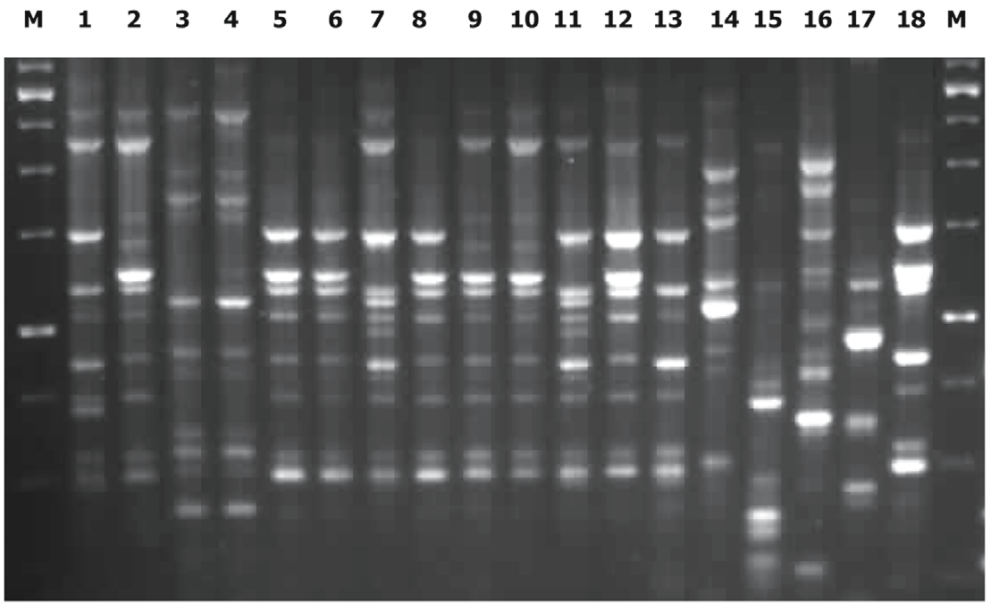

Fig. 1 Molecular typing of LAB by REP-PCR.
Lanes 1. K1, 2. K17, 3. K6, 4. K8, 5. K9, 6. K10, 7. K11, 8. K12, 9. K13, 10. K14, 11. K15, 12. K16, 13. K3, 14. K2, 15. K4, 16. K7, 17. L. lactis, 18. L. plantarum, M: 1 kb DNA ladder.

Table 1 Identification of *L. paraplantarum* and *L. pentosus* strains among the REP-PCR genotypes of LAB strains isolated from beetroot and carrot fermentation

LAB* strains	Source	REP-PCR genotypes	*recA* multiplex PCR	
			Amplicon size	Species
K1, K3	Beetroot	I	107 bp	*L. paraplantarum*
K17, K13, K14	Beetroot	II	107 bp	*L. paraplantarum*
K6, K8	Carrot	III	218 bp	*L. pentosus*
K9, K10, K12, K16	Beetroot	IV	107 bp	*L. paraplantarum*
K11, K15	Beetroot	V	107 bp	*L. paraplantarum*
K2	Beetroot	VI	218 bp	*L. pentosus*
K4	Carrot	VII	107 bp	*L. paraplantarum*
K7	Carrot	VIII	107 bp	*L. paraplantarum*

*Third day: K9, K10, K11, K12, K13, K14; sixth day: K15, K16, K17; 10th day: K1, K2, K3.

beetroot fermentation. LAB isolated on the 10th day of fermentation had a *L. paraplantarum* and *L. pentosus* genotype, which were different from the *Lactobacillus* strains isolated earlier. The LAB isolated from the traditional carrot Kanji revealed two genotypes of *L. paraplantarum* and one genotype of *L. pentosus* with the *L. pentosus* showing greater resemblance to that of the LAB strain K2 isolated from beetroot Kanji. The REP-PCR not only could aid in grouping the LAB strains, but was also useful for dissecting the strain variation among the members of *L. plantarum* group.

Discussion

The application of molecular techniques like REP-PCR, 16S rDNA sequencing and *recA* PCR has allowed the identification and characterization of the LAB associated with beetroot and carrot fermentation. The LAB strains analyzed in the present study showed similar ARDRA profile while DNA polymorphism could be detected among these strains by rep-PCR. Rep-PCR analysis of the LAB strains characterized in this study clustered them in two

distinct branches with *L. paraplantarum* isolates forming one cluster and *L. pentosus* isolates forming the other cluster (Fig. 2). The *L. plantarum* standard strain clustered along with the *L. paraplantarum* isolates whereas the *L. lactis* standard strain remained as a separate branch. Interestingly the *L. paraplantarum* (K4 and K7) and *L. pentosus* strains (K6 and K8) recovered from carrot Kanji differed from the LAB strains recovered from beetroot Kanji. The *L. plantarum*, *L. pentosus*, *L. parapalantarum* and the newly designated *L. plantarum* subspecies *argentoratensis*

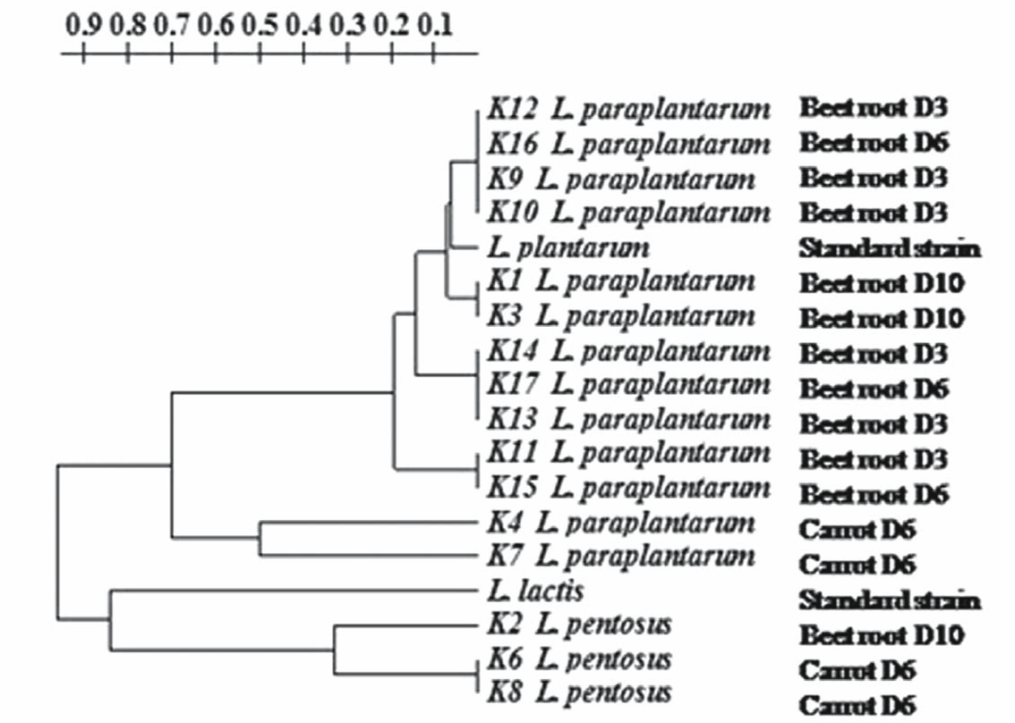

Fig. 2 Dendrogram showing the clustering of the LAB strains isolated from beetroot and carrot fermentation. Day of isolation is given after the source ie beetroot/carrot.

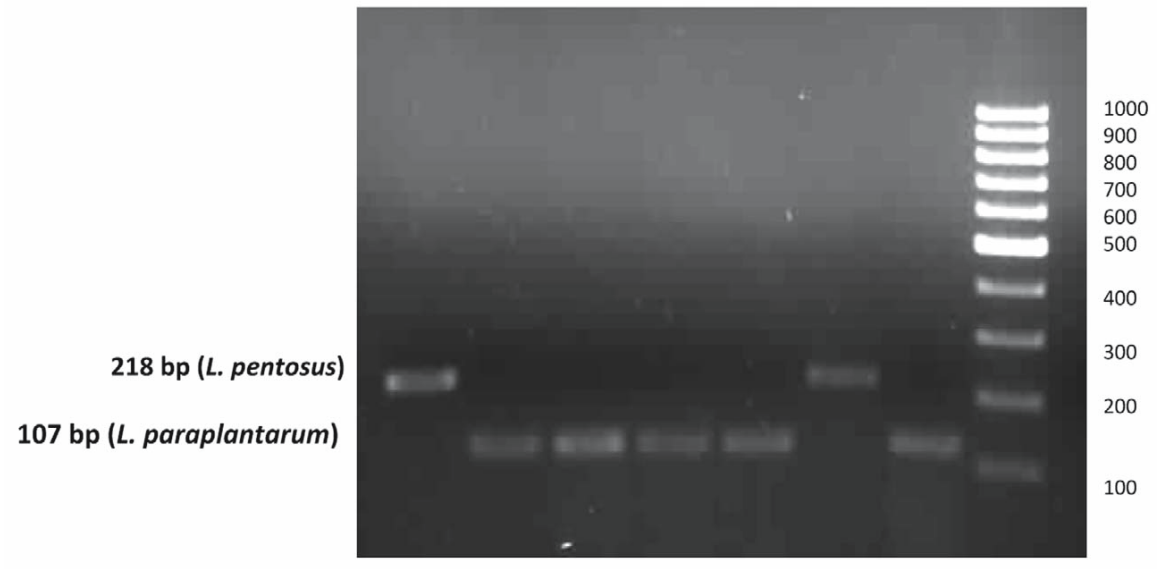

Fig. 3 Identification of *L. pentosus* and *L.paraplantarum* strains by *recA*-nested multiplex PCR assay.
L. pentosus: Lanes 1. K6, 6. K2.
L. paraplantarum: Lanes 2. K1, 3. K13, 4. K9, 5. K11, 7. K4, M: 100 bp ladder.

of the *L. plantarum* group share 99% sequence identity with respect to the 16S rDNA [15]. Hence 16S rDNA analysis by sequencing or restriction fragment length polymorphism (RFLP) of the LAB strains can at the most indicate, only a belonging to the *L. plantarum* group, but not to a definite species. In the present study as well, ARDRA and the partial sequencing of 16S rDNA could associate all the 16 LAB strains recovered, only to *L. plantarum* group. As a slowly diverging molecule, 16S rRNA is not able to reveal significant differences among recently diverged species such as *L. plantarum, L. paraplantarum* and *L. pentosus*, or *L. casei*, *L. rhamnosus* and *L. zeae* [16]. To obtain a more detailed identification, at species level, analysis of *recA* gene was undertaken, which has been described as a phylogenetic marker capable of differentiating the closely related members of the *L. plantarum* group [14, 15, 17]. The nested multiplex assay exploiting the variable nucleotide regions of *recA* gene proposed by Torriani et al. [14] revealed that 13 LAB strains which exhibited the 107 bp amplicon were of *L. paraplantarum* species whereas the remaining three LAB strains which showed 218 bp amplicon belonged to *L. pentosus* species.

The data generated from the above PCR-based assays suggest that community of LAB that dominate the beetroot fermentation is composed of a limited number of bacterial strains belonging to *L. paraplantarum* species. One *L. pentosus* strain was also isolated during sampling carried out at the completion of the beetroot fermentation. Three genotypes of *L. paraplantarum*, which formed the LAB community on the third day, could be seen during the sixth day also when the beverage was ready for consumption. On the 10th day a different genotype of *L. paraplantarum* not seen on the third or sixth day and a new *L. pentosus* genotype were recovered. It appears that a group of *L. paraplantarum* genotypes could be involved in this beetroot fermentation. A diverse array of *L. paraplantarum* strains are therefore available naturally and play a role in the fermentation, once the fermentation conditions are conducive for their growth. Earlier studies on the LAB of carrot Kanji using biochemical methods have identified them to be *L. mesentroides*, *Pediococcus* species and *L. dextranicum* [1]. In our observation, 16S rDNA sequencing followed by *recA* PCR-based detection approach has clearly indicated that the LAB associated with carrot/beetroot fermentation is *L. paraplantarum* and *L. pentosus*.

The results presented in this work provide insight in to the LAB population associated with Kanji fermentation. The LAB microflora associated with Kanji belongs to *L. plantarum* group as in much other vegetable fermentations. This strategy to identify the LAB strains involved in a fermentation process by REP-PCR and 16S sequencing followed by confirming them by a species-specific PCR would enable their identification in an unambiguous manner. Besides identification, the potential of REP-PCR to bring out the strain level variation would facilitate in selecting bacterial strains with desirable attributes for controlled fermentation or for their utilization in newer functional foods.

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