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Amrutha TA
Ph.D Scholar, Department of Dairy Microbiology, Verghese Kurien Institute of Dairy and Food Technology, Mannuthy, Kerala Veterinary and Animal Sciences University, Kerala, India

Ligimol James
Assistant Professor, Department of Dairy Microbiology, Verghese Kurien Institute of Dairy and Food Technology, Mannuthy, Kerala Veterinary and Animal Sciences University, Kerala, India

AK Beena
Professor and Head, Department of Dairy Microbiology, Verghese Kurien Institute of Dairy and Food Technology, Mannuthy, Kerala Veterinary and Animal Sciences University, Kerala, India

SN Rajakumar
The DEAN, Verghese Kurien Institute of Dairy and Food Technology, Mannuthy, Kerala Veterinary and Animal Sciences University, Kerala, India

Aparna Sudhakaran V
Assistant Professor, Department of Dairy Microbiology, Verghese Kurien Institute of Dairy and Food Technology, Mannuthy, Kerala Veterinary and Animal Sciences University, Kerala, India

Divya MP
Assistant Professor, Department of Dairy Chemistry, Verghese Kurien Institute of Dairy and Food Technology, Mannuthy, Kerala Veterinary and Animal Sciences University, Kerala, India

Corresponding Author:
Amrutha TA
Ph.D Scholar, Department of Dairy Microbiology, Verghese Kurien Institute of Dairy and Food Technology, Mannuthy, Kerala Veterinary and Animal Sciences University, Kerala, India

Assessment of techno-functional attributes of *Lacticaseibacillus paracasei* DMB11 isolated from Murrah buffalo milk for use as a functional starter culture for food fermentations

Amrutha TA, Ligimol James, AK Beena, SN Rajakumar, Aparna Sudhakaran V and Divya MP

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Abstract
The study aimed to assess the techno- functional attributes of *Lacticaseibacillus paracasei* DMB11, an indigenous lactic acid bacteria (LAB) isolated from Murrah buffalo milk for use as a functional starter culture in fermentation industry. On assessing its probiotic potential in terms of acid and bile tolerance the culture was found to be tolerant to pH 2 and 0.6% bile salts. Cell surface hydrophobicity (CSH) value with xylene was found to be 92%, and autoaggregation values were 27% at 1 h and 85% at 6 h. The coaggregation potential of the isolate against *Staphylococcus aureus* and *E. coli* were found to be 35.12% and 22.32%, respectively. The observations of this culture as non-haemolytic and negative for gelatin liquefaction indicated absence of virulence factors. Formation of glossy black colonies on Congo red agar indicated its ability for EPS production. The culture utilized citrate on growing in Kempler and Mckay media indicating its flavour production potential. Antioxidant property of the culture in terms of DPPH scavenging activity was found to be 46.61%. Fermented milk prepared by inoculating 1% culture, had an acidity of 1.19% and exhibited 6% syneresis. The findings of this study offer valuable insights into the possibility of application of *Lacticaseibacillus paracasei* DMB11 as a functional starter culture in fermented milk industry. However further studies are needed prior to the field or commercial level exploration of this culture.

Keywords: Lactic acid bacteria, fermented milk, *Lacticaseibacillus paracasei*

1. Introduction
The functional and aesthetic attributes of fermented milk products make them irreplaceable players in our food system. Sensorial as well as functional attributes of fermented products solely depend on the type of starter cultured used for the preparation. Nowadays, due to the increasing consumer demands for high quality and value-added functional food items, there has been a surge in the use of innovative functional cultures. Introduction of new food products with improved sensory attributes, health benefits, longer shelf lives and greater nutritional content is the primary thrust area in this direction. Novel starter cultures with functional attributes or 'functional starter cultures' are highly sought after for development of such innovative products. Functional starter cultures are "starters that possess at least one inherent functional property". The latter can contribute to food safety and/or offer one or more organoleptic, technological, nutritional, or health advantage [1]. Majority of the recognized 'functional starter cultures' belong to the lactic acid bacteria (LAB) group [2, 3]. This is quite understandable considering the wide use of LAB cultures as dairy starter cultures and probiotic cultures [4]. International Scientific Association for Probiotics and Prebiotics (ISAPP) defined "Probiotics are live microorganisms that when administered in adequate amounts confer health benefit in the host" [5]. *Lacticaseibacillus paracasei*, a member of LAB group is a Gram-positive bacterium commonly used in the fermentation of dairy products and has been found in the human intestinal tract and mouth.

Different strains of *Lacticaseibacillus paracasei* are being isolated from indigenous sources like animal gut, milk, vegetables, fruits and fermented foods and widely used as starter cultures for dairy products [6].

2. Materials and Methods

2.1 Starter culture

The *Lacticaseibacillus paracasei* DMB11 (NCBI accession number: OR905857) isolated from Murrah Buffalo milk was used in this work. For the experiments, the culture was activated by inoculation at one per cent level in reconstituted skimmed milk (10% TS w/v) and subsequent incubation at 37 °C till a count of minimum eight $\log_{10}$ CFU/ml was attained.

2.2 Technological Attributes

2.2.1. Activity Test

Activity of the culture was measured in terms of rate of acid production as described by Horrall and Elliker [7].

2.2.2 Citrate utilisation test

The citrate utilization potential was assessed by streaking the active culture on the media designed by Kempler and McKay [8] and subsequent incubation at 37 °C for 48 h. Development of bluish green colonies was taken as indicative of citrate utilization.

2.2.3 Exopolysaccharide (EPS) production

Ability of the isolate to produce EPS was determined by streaking it on Congo red agar and subsequent incubation at 37 °C. Formation of slimy and shining black colonies within 24 h of incubation was considered to be suggestive of EPS production [9].

2.3 Functional Attributes

2.3.1 Probiotic attributes

Invitro probiotic attributes of DMV11 were assessed based on ICMR-DBT guidelines [10].

2.3.1.1 Acid and bile tolerance

To assess the acid and bile tolerance, the culture was exposed to deMan Rogosa Sharpe (MRS) broth adjusted to pH (2.0 and 3.0) or added with 0.3 and 0.6% bile salt. Incubation was done at 37 °C [11]. Survivability of the isolate was qualitatively assessed by measuring the absorbance at 600nm of the culture broth at hourly intervals for a period of three hours.

2.3.1.2 Adhesion Potential

Auto-aggregation potential of the isolate was determined as per Kos *et al.* [12]. Freshly activated culture was centrifuged at 6000 rpm for 15 min at 4 °C, the cell pellet obtained was washed twice and resuspended in the phosphate-buffered saline (PBS) to an optical density of 0.6±0.02 at 600 nm. This cell suspension (0.1ml) and PBS (3.9 ml) were mixed well and the absorbance (A1) was measured at 600 nm. Samples was kept undisturbed for 1 h and 6h at 37 °C and the OD (A2) was determined at 600 nm. The auto-aggregation potential was calculated based on the following formula.

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

In the co-aggregation assay [13], bacterial suspensions for coaggregation were prepared in the same way as in auto-aggregation. *E. coli* and *S. aureus* (Raw milk isolates obtained from the culture collection of Department of dairy microbiology, VKIDFT, Mannuthy) were used as test organisms. The culture, test organisms and their mixture (1;1) were incubated at 37 °C for 1h and the absorbance was determined at 600 nm.

Coaggregation (%) = {[(AX+AY)/2] - [A(X+Y)]/[(AX+AY)/2]]} ×100

Where,
AX: Absorbance of culture
AY: Absorbance of test organism
A(X+Y): Absorbance of pathogen +culture mixture.

For cell surface hydrophobicity (CSH) test [13] by bacterial adhesion to hydrocarbons (BATH) assay, the cell pellets were suspended in PBS to an optical density of 0.25± 05 at 600 nm. To this suspension, an equal volume of xylene was added, mixed properly and OD of the mixture was recorded (A1). The mixture was kept undisturbed at 37 °C to allow phase separation. After 1h, OD of the aqueous phase was determined (A2).

CSH(%) :(A1-A2/A1) x100.

2.4 *In vitro* Safety assessment

2.4.1 Haemolysis and gelatin liquefaction

Haemolytic activity was determined by streaking overnight grown active culture on blood agar plates and subsequent incubation at 37 °C for 72 h. Incubation of activated bacterial culture streaked on gelatin agar slants for 7 days at 37 °C and observing for gelatin liquefaction after refrigeration for 1 h at 4 °C was done for assessing the gelatin liquefaction potential [14].

2.4.2 Antibiotic susceptibility

Minimum inhibitory concentration (MIC) of seven antibiotics (streptomycin, ampicillin, tetracycline, chloramphenicol, gentamycin, erythromycin and vancomycin) for the culture was determined using the EZY MIC ™ strips (Himedia, Mumbai) as per the manufacturer's instruction. Resistance/sensitivity was determined based on the interpretive data provided by the EFSA standards [15].

2.5 Antioxidant Activity - DPPH (2, 2-diphenyl-1-picrylhydrazyl) Assay

The antioxidant activity was measured in terms of DPPH scavenging activity as described by Son and Lewis [16]. Equal volume of the cell-free supernatant (CFS) of overnight MRS grown culture and freshly prepared 0.1 mM DPPH solution was mixed and kept for 20 minutes in dark room at room temperature. The absorbance of the mixture was measured at 517 nm. The radical scavenging activity was determined by the formula:

Radical scavenging activity (%) = [(Ac - As) / Ac] × 100

where
Ac = absorbance of the control (uninoculated MRS broth)
As = absorbance of the sample

2.6 Fermented Milk preparation

Milk (Homogenised toned cow milk) was subjected to boiling for 20 minutes. After cooling to 37 °C, it was inoculated with the culture at the rate of one percent and incubated at 37 °C till the pH reached to 4.5.

2.6.1 Acidity
The titratable acidity of the fermented milk was measured as per the standard method laid down by FSSAI [17].

2.6.2 Syneresis
Syneresis of the fermented milk was assessed as per the method described by Cartasev and Rudic [18]. The sample was weighed (W1) and kept at an angle of 45^0 for 20 min. The whey oozed out was removed and the sample was weighed again (W2).

% syneresis = [(W1-W2) / W1] x100

3. Results and Discussion
Lacticaseibacillus paracasei is a member of the normal human and animal gut microbiota and is used extensively in the food industry as starter cultures for dairy products and also as bacteria with probiotic features. Literatures suggest the use of *Lacticaseibacillus paracasei* isolated from different niches, as a starter culture in terms of both sensorial as well therapeutic attributes [6, 19].

3.1 Technological properties
3.1.1 Activity test
The rate of acid production of DMB11 was found to be 0.266% LA. As per the Horrall and Elliker [7] the starter cultures with activity below 0.3% LA are categorised as slow starters.

3.1.2 Citrate utilization
DMB11 showed a positive reaction (Fig 1b), on Kempler and Mckay agar indicating their citrate utilization ability. Citrate utilization potential point to the ability of the culture to produce aroma compounds like acetate, diacetyl, acetoin, and 2,3-butanediol, important flavouring compounds in fermented milk. Presence of citrate utilizing LAB in fermented milk will definitely influence its sensory profiles and increase its acceptability among the consumers [20].

3.1.3 EPS production
Formation of slimy type transparent colonies in MRS agar are considered indicative of the ability of *Lacticaseibacillus paracasei* DMB11 to produce exopolysaccharide (EPS). As shown in Figure 1a, DMB11 formed black colonies on Congo red agar establishing its EPS production capability.
Exopolysaccharide production is a desirable feature for bacteria used in dairy products because EPSs act as natural bio thickener leading to higher consistency, viscosity and reduced syneresis of the product. Most of the currently used bio thickeners are chemically or enzymatically modified ones and, therefore, their use is not encouraged for food applications. An alternative source of biopolymers is microbial EPS. EPS may induce positive physiological responses including immunomodulation, lower cholesterol levels, reduce formation of pathogenic biofilms, modulate adhesion to epithelial cells, and increase levels of commensal microbes in host [21, 22]. Hence, the use of EPS-producers seems to be adventitious over nonproducing starter cultures.

3.2 Probiotic characterization
For a bacterial culture to be termed as a probiotic, it must be able to survive in the hostile gastric environment. The acidic pH (1-2) in stomach is the foremost challenge for the probiotic bacteria to reach their target site. As the ingested food buffers the acidic environment of stomach to pH 3, this pH is considered as the optimum pH for testing the survivability of a potential probiotic strain [23]. The results of *in vitro* assessment of the DMB11 strain showed good tolerance to 0.3% bile salt, pH 2.0 and 3.0 (Fig 2) and remarkable adhesion potential (Table 1), indicating the probiotic potential of *Lacticaseibacillus paracasei* DMB11.

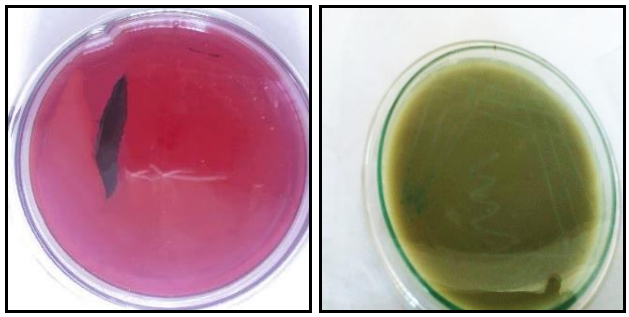


Fig 1: a) EPS production-Black shiny colonies on CR agar
b) Green colonies on Kempler and Mckay media

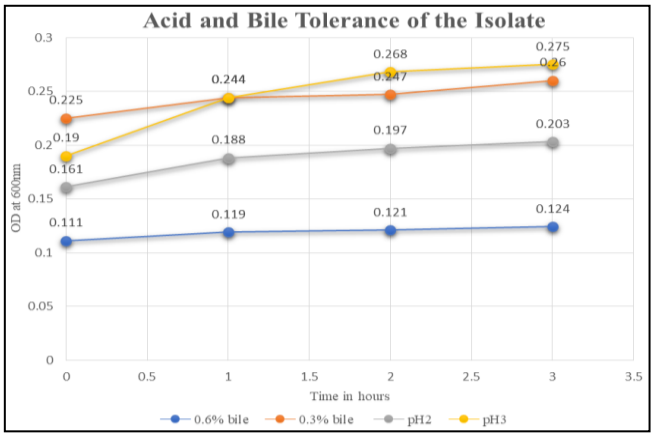


Fig 2: Acid and bile tolerance of *Lacticaseibacillus paracasei* DMB11

Table 1: Adhesion properties of *Lacticaseibacillus paracasei* DMB11

Property		% of adhesion
Cell surface hydrophobicity		92± 0.47%
Auto aggregation	1h	27± 0.81%
	6h	85± 0.45%
Coaggregation	*S. aureus*	35.12 ± 0.54%
	E. coli	22.32±0.41%

The propensity of a microbial strain to adhere on the epithelial cells can be measured in terms of adhesion potential. Auto aggregation, coaggregation and BATH assays are the key qualitative phenomenological approaches for estimating adhesion potential. Adherence of bacterial cells is usually related to cell surface characteristics. Cell surface hydrophobicity, the nonspecific interaction between host and microbial cells is an important cell surface property that influences the adhesion of bacteria to different surfaces. Based on the CSH value the hydrophobicity of the bacterial strains are categorised into low (< 33%), medium (33% - 66%), and high (> 66%) [24]. Highly hydrophobic bacterial cells interact strongly with mucosal cells. The CSH value of the isolate in this study was 92.42% which is high when compared to the CSH value of *Lactobacillus* strains reported by Zommara *et al.* [25]. According to Wang *et al.* [26], an auto aggregation value higher than 40% is exceedingly good. A high auto aggregation value of 83.78% was showed by DMB11.

The coaggregation potential of *Lacticaseibacillus paracasei* DMB11 against *S. aureus* and *E. coli* was found to be 35.12% and 22.32% respectively. Coaggregation is considered as a key strategy adopted by *Lactobacillus* strains to prevent pathogenic bacteria from adhering to biological surfaces through competitive exclusion. Zhou *et al.* [27] studied the coaggregation potential of *L. paracasei* ZFM 54 against *H. pylori*. Barache *et al.* [28] reported the coaggregation potential of *L. paracasei* FB1 with *S. aureus* (28.34%), *E. coli* (23.39%) and *L. monocytogenes* (20.10%). Agreeing with this report, *Lactobacillus paracasei* DMB11 exhibited higher coaggregation potential with *S. aureus* than that with *E. coli.* Ability of a bacteria to produce exopolysaccharide has been shown to stimulate their adhesion potential to intestinal mucosa [29]. So, its EPS production ability might have contributed to the high adhesion potential of DMB11 observed in the current study.

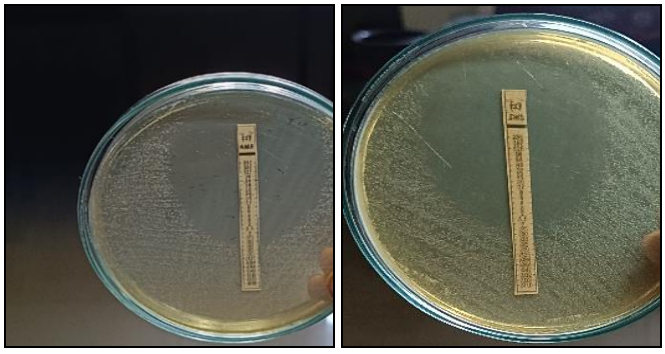
3.3 Safety assessment

The safety studies found that *Lacticaseibacillus paracasei* DMB11 has no haemolytic, gelatin liquifying nature and can be considered safe as reported for *L. paracasei* GMNL-33 and *L. paracasei* L1 [30, 31].

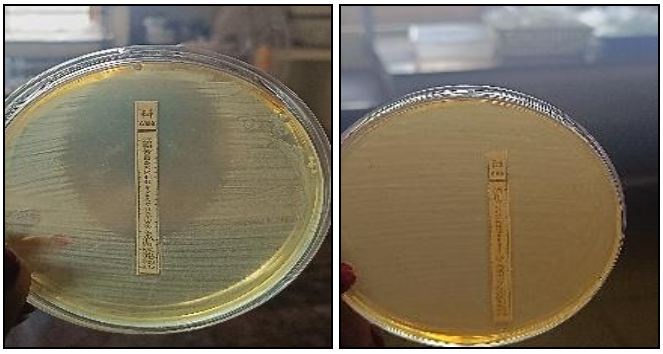
Table 2: MIC determination of *Lacticaseibacillus paracasei* DMB11

Antibiotic	Breakpoint by EFSA (mcg)	MIC
Ampicillin	4	0.38
Chloramphenicol	8	1
Gentamicin	16	4
Vancomycin	NR	0
Tetracycline	8	8
Amoxycillin	NM	0.125
Streptomycin	64	48
Erythromycin	1	12

NR: Not required NM: Not mentioned in EFSA



a) Ampicillin b) Chloramphenicol



c) Amoxycillin d) Vancomycin

Fig 3: Determination of MIC values using antibiotic strips

MIC values of the tested eight antibiotics against *Lacticaseibacillus paracasei* DMB11 are shown in table 2 and Fig.3. For all the tested antibiotics, except erythromycin *Lactobacillus paracasei* DMB11 exhibited MIC values lower than the MIC breakpoint values recommended by EFSA for heterofermentative *Lactobacillus paracasei*. Agreeing with the previous reports of intrinsic resistance of *Lactobacillus sp*. to vancomycin [32, 33]. *Lactobacillus paracasei* DMB11 was found to be resistant to this antibiotic. Observations of the current study are in support of the reports of erythromycin resistance of *Lactobacillus* strains [34, 35].

3.4 Antioxidant activity

The DPPH scavenging activity of DMB11 was found to be 46.61±0.39%. Won *et al.* [36] reported that *Lacticaseibacillus paracasei* MG5004 and *Lacticaseibacillus paracasei* MG5012 showed DPPH scavenging potential 60.8 ± 0.8 and $32.6 \pm 6.1\%$, identifying that the ability to scavenge the DPPH radical as a strain-dependent feature in LAB. DPPH scavenging is one of the most important indicators for determining the antioxidant activity of LAB *invitro* [37]. Probiotic strains possessing antioxidant properties can be a support in balancing homeostasis and reducing the progress of several diseases. Also, they help to prevent the unwanted oxidation reaction in the food products thereby increase the shelf life of the products. The use of LAB strains with effective antioxidant systems can stabilize the free radical levels and may contribute to immunomodulatory effect [38, 39].

Fig 4: Fermented Milk prepared using *Lactobacillus paracasei* DMB11

3.5 Fermented milk characteristics

Fermented milk prepared using DMB 11 had a soft glossy appearance with creamy texture (Fig.4). The acidity of the product prepared was found to be 1.1% LA. Syneresis of the prepared fermented milk was six% at pH4.5. In spite of its grading as a slow starter by the Horrall Elliker test, DMB11 was found capable to produce a fermented milk with acidity higher than 0.45% LA, the minimum acidity level stipulated by FSSAI [40] for a fermented milk product. From the industrial point of view, the key attributes required for a fermented milk product are the acidity and low level of syneresis. Product prepared using the strain DMB11 was found to exhibit these attributes.

Results of this study endorse the techno-functional suitability of the *Lactobacillus paracasei* DMB 11 as a functional starter culture in dairy fermentation industry. This strain exhibited good adhesion properties and antioxidant activity. However future studies are needed before its commercial exploration for the development of novel dairy- or non-dairy functional products.

4. Conflict of Interest

Not available

5. Financial Support

Not available

6. Reference

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