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Probiotic characterisation of *Enterococcus faecalis* strain isolated from a household dahi sample of Wayanad district, Kerala

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Abstract

Household dahi sample with good organoleptic properties from Wayanad district of Kerala was screened for lactic acid bacteria. Gram positive cocci exhibiting potential to ferment lactose was further characterised biochemically and later identified by 16srRNA sequencing as Enterococcus faecalis. The isolate was evaluated for its physiological and probiotic properties like temperature tolerance, salt tolerance and proteolytic activity. Probiotic properties like acid tolerance, bile tolerance, adhesion potential and antimicrobial activity against clinical isolate of E. coli were evaluated. Virulence based on haemolysis in blood agar was also done. The isolate was able to survive both 4°C and 45°C as well as salt concentration of 2 to 8 per cent. This remarkably proteolytic isolate was able to survive at pH 2 and 3 for 3 h and could tolerate bile concentration of 0.3 per cent for 3 h and 0.6 per cent for 1 h. Cell surface hydrophobicity was found to be 19.6 per cent and auto aggregation 80.39 per cent. The isolate could inhibit the growth of pathogenic E.coli by producing a zone of clearance of 19mm. Antibiogram revealed resistance to Ceftriaxone, Cefotaxime, Cefazolin and Vancomycin. The isolate produced alpha haemolysis in blood agar. Study revealed that detail biochemical and molecular level characterization is required for assessing probiotic potential of lactic acid bacteria from naturally fermented products.

Keywords: *Enterococcus faecalis*, *dahi, probiotics*

Dahi ('Thairu' in local parlance) is a popular indigenous fermented milk product which is regularly prepared and propagated in the house holds of India. Other than being a standalone side dish, it is also an indispensable component in many traditional Indian cuisines (Prajapati and Nair, 2003). The sensory characteristics of dahi is mostly attributed to the mixed, undefined flora

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of starter culture that goes into the making of curd by back slopping in each household. Dahi exhibit wide differences in acidity, flavour, and texture between households due to variation in quality of milk, type of starter culture, temperature, time and storage conditions (Coggins *et al.*, 2010). Microflora of household dahi is generally dominated by *Lactococci, Streptococci, Lactobacilli, Enterococci* and lactose fermenting yeast. Many members of lactic acid bacteria especially those belonging to *Lactobacillus* and *Bifidobacterium* are considered as probiotics and are being used industrially in fermented foods. Nowadays, lactic acid bacteria like *Enterococcus*, *Aerococcus* and *Carnobacterium* are also being explored as probiotic candidates due to their technological advantages (Franz *et al.*, 2011).

Enterococcus is a gram positive facultative anaerobic bacterium found in diverse environmental niches including human gut. *Enterococci* are gaining importance in food industry owing to its potential to survive extreme conditions like high salt concentrations, ability to multiply at wide range of temperatures, production of proteolytic and lipolytic enzymes, and probiotic attributes like acid tolerance, bile tolerance and adhesion potential (Haghshenas *et al.*, 2017). They are also known to produce antibacterial polypeptides such as enterocins which are useful in biopreservation (Franz *et al.,* 2007). *Enterococcus faecalis, E. faecium* and *E. durans* are the common enterococci reported to be isolated from traditional fermented dairy products as well as non-dairy products (Nami *et al.*, 2019). However certain strains of enterococci are reported to possess virulence factors (Economou *et al.*, 2017) and genes for antimicrobial resistance (Almeida *et al.,* 2020). Enterococci are intrinsically resistant to many antimicrobials (β-lactams and cephalosporins) and can easily acquire high-level drug resistance *via* horizontal gene transfer. Vancomycin Resistant Enterococci (VRE) play an important role in the inter and intraspecies transfer of antimicrobial resistance genes (Giraffa, 2002).The present study attempted to characterise the dominating lactic acid bacteria in an organoleptically superior dahi sample collected from the Wayanad district of Kerala.

Materials and methods

Sample collection

Dahi samples from 10 households in Wayanad district were organoleptically evaluated by a panel comprising of untrained judges. The sample which got highest score was used for further study. Serial dilutions were prepared in normal saline (0.9 per cent sodium chloride) and plated in de Man Rogosa Sharpe Agar (MRS) and incubated at 37ºC for 48h to get well discrete colonies. The carefully selected colonies were streaked to purity and purified culture (ADMH 19) was maintained in MRS agar slants at 4ºC. For long time preservation, the pure culture was stored in 70 per cent glycerol at -80ºC.

Isolation and identification

Primary identification tests like Gram staining, catalase test, oxidase and IMViC tests as per Barrow and Feltham (1993) were done. Potential of the isolate to ferment sugars; lactose, rhamnose, cellobiose, mannose, trehalose, raffinose, galactose, melibiose, arabinose, inositol and adonitol were determined in Andrade peptone water (MacFaddin, 1985). Molecular level confirmation of the isolate was done by 16S rRNA sequencing. The primers used were 27F (5'AGAGTTT GATCCTGGCTCAG 3') and 1492R (5'GGTTACCTTGTT ACGACTT 3') (Pang *et al.*, 2012). Sequence analysis was performed using online tool BLAST of NCBI database. Based on maximum identity score E value, top most sequences were utilised for multiple sequence alignment (Clustal W2). Phylogenetic tree was constructed using Neighbour joining method in MEGAX.

Temperature and salt tolerance

Growth of isolate at different temperature was measured as described by Kanasaki *et al.* (1975). Sterile MRS broth was inoculated with freshly activated culture at 2 per cent level and incubated at different temperatures *viz.* 4ºC, 10ºC, 25ºC, 37ºC and 45ºC. After overnight incubation (18h) the optical density was measured at 600 nm. Salt tolerance was measured by inoculating freshly activated culture at the rate of 2 per cent to

MRS broth containing sodium chloride at two, four, six and eight per cent level and incubated at 37ºC for 6 h. Optical density was measured at 600nm and salt tolerance determined as described by Briges, (1953).

Proteolytic activity

The proteinase activity of the isolate was assessed qualitatively by streaking them on skim milk agar (Nutrient agar with 10% skim milk) followed by incubation at 37ºC for 48 h (Harrigan, 1998). Development of zone of clearance around the colonies was considered as positive test for proteolytic activity.

Gut survival studies

Acid and bile tolerance: The MRS broth was inoculated with one per cent culture and incubated at 37ºC for 18h. The acid tolerance of isolate was assessed by exposing the isolate to pH 2.0 and pH 3.0 by adjusting the pH of MRS broth using 1N HCl. The growth at low pH was qualitatively assessed by streaking on MRS agar plates at 0h, 1h, 2h and 3h of incubation at 37ºC. The bile tolerance was assessed by inoculating the isolate to MRS broth containing 0.3 and 0.6 per cent bile salt and incubated at 37ºC for 4h to simulate the transition time in intestine. The growth at different bile concentration were qualitatively assessed by streaking on MRS agar plates after 0h,1h,2h and 3h of incubation at 37ºC (Pundir *et al.,*2013).

Adhesion potential

Adhesion potential of isolate was evaluated based on the auto aggregation (%) and cell surface hydrophobicity. Autoaggregation was measured as per the procedure described by Del Re *et al*. (2000). The MRS broth was inoculated with one per cent culture and incubated at 37ºC for 18h, The cells were harvested by centrifugation at 5000g for 15 min. The cell pellets were washed twice in phosphate buffered saline (PBS) and then resuspended in PBS to a final optical density of 0.6 at 600nm. A total of four millilitres of this cell suspension was vortexed and 0.1ml was transferred to 3.9 ml of PBS and kept undisturbed at 37ºC for 6 h. The optical density of upper suspension was measured after 6h at 600nm. Auto aggregation was then calculated as

Autoaggregation % = [1-(A1/A0) x100] where A0 and A1 are the initial and final absorbance values

Hydrophobicity was measured as per the procedure described by Rosenberg *et al.*, (1980). Overnight grown cultures were centrifuged at 5000g/15 min and the cell pellets were washed twice in PBS. The pellets were then resuspended in 3 ml of PBS and equal volume of xylene was added. After thorough mixing the optical density was measured at 600nm. The sample was kept undisturbed at 37ºC for 1h and optical density of aqueous phase was measured at 600 nm. Hydrophobicity (%) was calculated as follows:

$H\% = [(A0 - A)/A0] \times 100$, where A0 and A are the initial and final absorbance values.

Antimicrobial activity

The antimicrobial activity of isolate against *E. coli* (pathogenic isolate from a case of canine diarrhoea from the Department of Veterinary Public Health, CVAS, Pookode) was done by agar well diffusion assay (Tagg and McGiven, 1971).To evaluate the antimicrobial effect of the isolate, the overnight grown culture was centrifuged at 10000g for 15 min at 4ºC and filter sterilised (0.45um Millipore filtre). A total of 0.1 ml of *E. coli* culture (10^6cfu/ml) was spread on Nutrient agar plates and a well (8mm diameter) was punched in the plate, 100ul of cell free supernatant was filled in the well and the plate was kept upright at 37ºC for 24 h. Inhibition zone diameter around the well was measured at the end of incubation.

Antibiotic susceptibility testing

Antibiotic susceptibility pattern was determined by standard disc diffusion method (Bauer *et al.*, 1966). Amoxicillin(10mcg), Cefoperazone (75mcg), Tetracycline (30 mcg),Ceftriaxone (30 mcg), Azithromycin (15 mcg), Ampicillin(10 mcg), Co-Trimoxozole (25 mcg), Levofloxacin (5 mcg), Amikacin(30

mcg), Amoxyclav (30 mcg), Chloramphenicol (30mcg), Netillin (30mcg), Ofloxacin (5 mcg), Ciprofloxacin (5 mcg), Nitrofurantonin (300 mcg), Gentamicin (10 mcg), Cefotaxime (30mcg), Cefazolin (30 mcg), Linezolid (30 mcg), Doxycycline Hydrochloride (30 mcg) and Vancomycin (30 mcg) were tested. After incubation at 37ºC for 24 h inhibition zones were measured. Size of inhibition zone was compared with the zone size interpretation chart of manufacturer (Himedia).

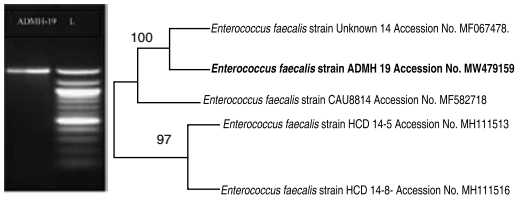
Haemolytic activity

The isolate was streaked on brain-heart infusion agar containing 5per cent of sheep blood. After incubation at 37ºC for 48 h, plates were examined for zone of clearance that were suggestive of haemolysis (Semedo *et al.*, 2003).

Results and discussion

Isolation and identification of Enterococcus faecalis

The isolate was Gram-positive, cocci that was catalase and oxidase negative and arranged as chains or diplococcic. The isolate obtained in this study could ferment Arabinose, Melibiose, Galactose, Raffinose, Lactose, Cellobiose, Rhamnose, Mannose, Trehalose, Adonitol and Inositol. IMViC reaction was found to be -+--.The sequence analysis of 16S rRNA gene confirmed the isolate to be *Enterococcus faecalis (*Fig.1a). The sequence was deposited in NCBI and assigned with accession number MW479159. The phylogenetic tree of the isolate ADMH 19 was constructed (Fig.1b). The isolate ADMH 19 showed 100 per cent similarity to *E. faecalis* MF067476 isolated from salted fish (United Arab Emirates). *Enterococcus fecalis* has been isolated from homemade dahi in various states of India as well as other countries in the Indian subcontinent (Shangpliang *et al.,* 2017). Harun-ur-Rashid *et al.* (2007) could isolate enterococcus in nine percent of dahi samples in Bangladesh. However, the data regarding the abundance of this organism in dahi is by and large scarce in our state. Prevalence of *E. faecalis* in dahi prepared using undefined starters need to be explored in detail in the state.



(a) (b)

Fig. 1 **(a).** PCR amplification of 16S rRNA using universal primer 27F and 1492 R

(b). Phylogenetic tree constructed using Neighbour joining method in MEGAX.

Tolerance to temperature and salt

The isolate was able to grow at the temperature ranging from 4 ºC to 45 ºC with maximum growth at 37 ºC. The optimum, minimum and maximum temperatures for *E. faecalis* on brain heart infusion (BHI) agar in aerobic conditions were reported to be 42.7, 6.5 and 47.8 ºC, respectively (Van den Berghe *et al*., 2006). Wessels *et al*., (1990) reported that *Enterococcus faecalis* isolated from dairy products were able to grow at psychrotropic temperature of 7 ºC. This could be due to the membrane structure of *Enterococcus* sp which was more stable at lower temperature and less resilient at higher temperatures (Ivanov *et al*., 1999). The isolate was able to survive at two four, six and eight per cent salt concentrations with maximum growth at two per cent level. The ability to survive at higher salt concentration can be attributed to the cation homeostasis (Fisher and Philip, 2009). Also the isolate was found to phylogenetically more similar to *E. faecalis* isolated from salted fish as mentioned earlier. The potential of the isolate to grow at both psychrophilic and thermoduric temperatures along with the salt tolerance can be potentially exploited in food fermentations like cheese ripening and lactic acid production (Yuan *et al.*, 2018; Sun *et al.,* 2020).

Proteolytic activity

The isolate produced clear zone around the colonies showing its proteolytic activity (Fig. 2). Hydrolysis of milk protein casein plays an important role in development of flavor, texture and also release of bioactive peptides.

Enterococcus faecalis have been reported to be remarkably more proteolytic when grown on dairy substrate than other enterococcal species due to the presence of *gelE* gene (Morandi *et al*., 2006). Enterococci are usually detected in cheese during initial days of ripening and this could be due to their ability to produce proteases and peptidases (Settanni *et al*., 2010). Although the proteolytic activity is beneficial in cheese ripening, the presence of virulent genes like *gelE* in *E. faecalis* is restricting their use in cheese as a nonstarter lactic acid bacteria. But the purified peptides produced by this isolate could be used in formulations.

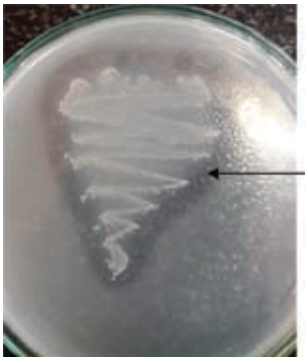


Fig. 2. Proteolytic activity of *E. faecalis* ADMH 19 in skim milk agar

Gut- survival studies

The ability to survive in the acidic gastric environment and resistance to bile salts are important determinants of a probiotic. Tolerance to gastric acidity (pH 2.0-2.5) is considered as a functional requirement of a probiotic (Bevilacqua *et al*., 2010). The isolate in this study could tolerate the acidic pH of 2 and 3 for 3 h with less dense growth at pH 2. Even though the isolate survived a bile concentration of 0.3% for 3h it was unable to survive at 0.6% for more than 1 h (Fig. 3 and 4). Baccouri *et al.,* (2019) reported a survivability of 84 to 97 per cent for *E. faecalis* isolates at pH 3 after 3 h of incubation. Klaenhammer and Kullen, (1999) reported that when compared to other lactic acid bacteria *Enterococcus sp* exhibited higher bile tolerance.

Cell surface hydrophobicity and autoaggregation ability are two independent traits that determine the adhesion ability of bacteria to biotic and

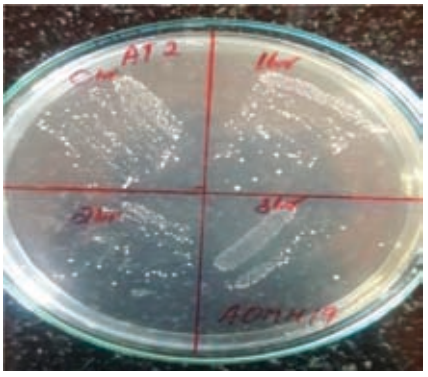


Fig: 3. Acid tolerance of ADMH 19 at pH 2.0 for 0h,1h,2h and 3h

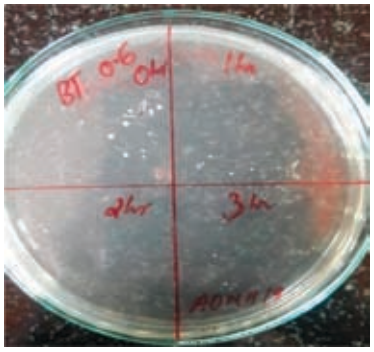


Fig: 4. Bile tolerance of *E.faecalis* ADMH 19 at 0.6 % bile for 0h,1h,2h and 3h

abiotic surfaces. The affinity of enterococcal isolate to hydrophobic solvent xylene was observed and it was found that cell surface hydrophobicity was 19.6 per cent and auto aggregation of 80.39%. Several studies have reported a positive correlation between these two traits in determining adhesion ability (Del Re *et al*., 2000). Serrano-Niño *et al*. (2016) opined that a CSH value above 50 per cent indicated high hydrophobicity. According to Zommiti *et al*. (2018), strains with an autoaggregation of more than 40 per cent could be graded as good and those with a value less than 10 per cent, as weak. The CSH value in our study was found to be comparatively lower but the auto aggregation ability was found to be > 80 per cent. Few studies have reported heterogeneity in cell surface hydrophobicity as it was influenced by several factors interfering the adhesion of cells to hydrocarbons like cultivation time, composition of cultivation media, presence of some acids and the type of solvent used (Busscher *et al*., 1995).

Antimicrobial activity of *E. faecalis* isolate against clinical *E. coli* was conducted and a clearance zone of 19 mm was observed (Fig.5). Several species of enterococci are reported to inhibit both gram positive and gram negative bacteria (Zheng *et al*., 2015). Many strains of *E. faecalis* have been reported to produce bacteriocins and are considered as potential probiotics by eliminating pathogens (Al Atya *et al.*, 2015). Since we have not confirmed the presence of bacteriocins, the inhibitory action could also be attributed to other factors like production of organic acids and hydrogen peroxide.

Antimicrobial resistance contributes to serious public health concerns. The isolate was sensitive to Amoxicillin, Azithromycin, Ampicillin, Levofloxacin, Amoxyclav, Chloramphenicol, Netillin, Ofloxacin, Ciprofloxacin, Nitrofurantonin, Gentamicin, Linezolid and Doxycycline Hydrochloride. The resistance to Cephalosporins and Vancomycin was observed in our study. *Enterococcus faecalis* and *E. faecium* were reported to be naturally resistant to cephalosporins, aminoglycosides (low-level resistance), macrolides, and sulphonamides, also clindamycin and quinupristin/dalfopristin (EUCAST, 2016). Vancomycin resistance in *E. fecalis* has been documented by Cetinkaya *et al*., (2000). The vancomycin resistance genes of enterococci are transposons or 'jumping genes' located in the plasmids and hence enterococci have been reported to serve as donors of vancomycin resistance gene clusters to more pathogenic microorganisms such as MRSA (Ray *et al*., 2003) The resistance to vancomycin is of special concern due to the important therapeutic use of these agents against the MDR enterococci and other Gram-positive bacteria (Hollenbeck and Rice, 2012).

Haemolytic activity

Establishing the safety of bacteria to be used as a probiotic requires it to be non haemolytic (Oh and Jung, 2015). Even though no β-hemolytic activity was detected in the isolate, greenish zones were observed in blood agar, indicating α-hemolytic activity (Fig. 6). Presence of haemolytic activity indicated that the isolates could be potentially pathogenic. Findings

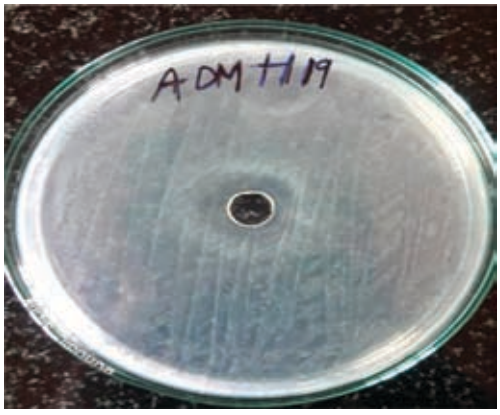


Fig. 5. Agar well assay of *E. faecalis* ADMH 19 showing a zone of inhibition against pathogenic *E.coli*

Fig. 6. Haemolytic activity of *E. faecalis* ADMH 19 showing zone of alpha haemolysis in sheep blood agar

contradict with Nami *et al.*, (2019) who reported the absence of haemolytic activity in *E. faecalis* strains isolated from artisanal dairy products. Presence of α-hemolytic activity necessitate the need for molecular level studies to rule out the presence of virulence genes before confirming the safety

The observations in this study confirm the high endurance of the isolate *Enterococcus faecalis* ADMH 19 to various environmental stresses. Acid and bile tolerance, antimicrobial activity and auto aggregational features support its potential to survive in the gut. However, the partial haemolysis and resistance to new generation antibiotics raise serious concern.

The isolation of MDR lactic acid bacteria from house-hold curd warrant special attention from public health point of view.

Conclusion

Enterococcus faecalis ADMH19 isolated from household curd was found to fulfil the probiotic criteria like acid tolerance, bile tolerance and adhesion potential. The isolate also exhibited antimicrobial activity against pathogenic *E.coli*. However, due to the association of some enterococci to human infection further studies on virulence factors and multiple drug resistance are required to ensure safety of *E.faecalis* isolated in this study.

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Conflict of interest

The authors declare no conflict of interest.

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