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Antibiogram of *Enterococcus sp.* isolated from household *thayir* sample#

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Abstract

The present study aimed to evaluate the antibiotic susceptibility of Enterococcus sp. isolated from a household dahi/thayir sample. Bile esculin agar was used for the selective isolation of Enterococcus sp. Based on biochemical characterization and 16SrRNA sequencing, the isolate was identified as Enterococcus faecium, the sequence of which is deposited in NCBI with accession number OR105180. Enterococcus faecium was found to be resistant to Penicillin, Ampicillin, Vancomycin, Tetracycline, and Erythromycin. Multiple Antibiotic Resistance (MAR) index of the isolate was found to be 0.625. Minimum inhibitory concentration for Penicillin, Ampicillin, Vancomycin, Tetracycline and Erythromycin exceeded the break point established by CLSI guidelines. Microtiter plate assay revealed the isolate to be a weak biofilm former. Safety assessment revealed the isolate to be alpha haemolytic with no gelatinase activity. The observations endorse the possibility of household dahi/thayir samples being a potential reservoir for the dissemination of antibiotic resistance to human gut microflora.

Keywords: *Enterococcus faecium, antibiotic resistance, multiple antibiotic resistance (MAR) index*

Antibiotic resistance has emerged as the major public health problem facing humanity globally. World Health Organization (WHO) reports state that antibiotic resistance in bacteria causes more than seven lakh deaths globally and this may increase to ten million by the end of 2050. Antibiotic resistance in the food supply chain is a serious concern but often overlooked especially in developing countries as there are no immediate consequences. Due to the same

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reason, this remains as a concealed threat in food chains (Founou *et al.,* 2016) With the propounded health benefits of fermented milk products, there has been a clear shift towards the inclusion of these products in diets. Fermented milk products that contain trillions of starter organisms can be equally responsible for introducing antibiotic-resistant bacteria to the human gastrointestinal tract disseminating genes for antibiotic resistance (Sharma *et al.,* 2014).

Enterococcus, a genus coming under lactic acid bacteria, is often encountered in fermented milk products and is now at a crossroads in food safety, due to reports of multiple drug-resistant traits in them. With their innate potential to tolerate wide environmental conditions, enterococci remain ubiquitous and persist in a wide range of ecological niche enabling their spread through the food chain. Enterococci, which possess multiple drug resistant traits, have been identified as one of the most common aetiology for nosocomial infections and their presence in food products is a real concern (Hanchi *et al.,* 2018). *Dahi/Thayir*, the indigenous fermented milk product prepared by fermentation of milk using lactic acid bacteria is a regularly consumed dietary staple in India. The present study attempted to evaluate the antibiogram of *Enterococcus sp.* isolated from household curd/*thayir* samples.

Materials and methods

Isolation of Enterococci sp.

Aseptically collected *thayir* sample, after appropriate dilution, was pour plated in Bile esculin agar (Himedia) for the isolation of enterococci. Esculin hydrolysing organisms evident as black hallowed colonies, presumptive for enterococci, were subjected to preliminary characterization (Gram staining, Catalase, and Oxidase test) as per the standard procedure described by Barrow and Feltham, (1993). The isolate was streaked to purity and stored in De man Rogosa and Sharpe (MRS) agar slants at 4°C. The potential of the isolate to utilize different carbon sources was determined using Andrade Peptone Water following manufactures' guidelines (Himedia, Andrade Peptone Water-LQ174). The colour change from pale tan to deep pink was indicative of acid production. Motility of the isolate was tested as per the procedure described by Ball and Sellers, (1966) using 2,3,5- triphenyl tetrazolium Chloride (TTC). The freshly activated culture was stabbed into TTC (1%) incorporated soft nutrient agar (with 0.8% agar) and incubated at 37^{0}C for 24h. Spreading of growth from the line of stab was considered as indicative of the motile nature of the isolate. Physiological and biochemical characteristics of the isolate were evaluated.

Molecular level confirmation of the isolates was done by 16SrRNA sequencing by outsourcing to Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram. The primers used were 16S-RS-F Forward 5'CAGGCCTAACACATGCAAGTC3' and 16S-RS-R Reverse 5'GGGCGGWGTGTACAAGGC3' (Jacob *et al.,* 2017).

Antibiotic susceptibility of the isolate by Disk-diffusion assay

Antibiotic susceptibility of the isolate was evaluated by the disk diffusion assay as per Bauer *et al.* (1966). Antibiotics selected were Penicillin (10 units), Ampicillin (10 mcg), Linezolid (30 mcg), Vancomycin (30 mcg), Tetracycline (30 mcg), Chloramphenicol (20 mcg), Erythromycin (15 mcg) and Rifampicin (20 mcg). Antibiotics were selected and the results were interpreted based on guidelines proposed by the Clinical and Laboratory Standards Institute (CLSI), 2020.

Multiple Drug Resistance index (MAR index) was calculated using the following equation (Krumperman, 1983)

MAR index = a/b

Where "a" represents the number of antibiotics to which isolates were resistant,

"b" represents the number of antibiotics to which isolates were exposed

Minimum inhibitory concentration (MIC) of the isolate

The Minimum inhibitory concentration of Penicillin, Ampicillin, Linezolid,

Vancomycin, Tetracycline, Erythromycin and Chloramphenicol were determined by the MIC strip (Himedia, Ezy MIC™ Strip) following manufactures' guidelines. Suspension of enterococcal isolate with an optical density adjusted to 0.5 McFarland standard was swabbed in Muller Hinton Agar plates. Sterile MIC strips with antibiotic gradients were placed on the agar and incubated at 37°C for 24h. MIC values were read at the point of intersection of the zone edge and the MIC strips.

Biofilm formation by congo red assay

Biofilm forming potential was qualitatively evaluated based on colony characteristics when streaked on Congo red agar (Freeman *et al.,* 1989). Congo red agar was prepared by adding 0.1 percent Congo red solution at a level of nine percent to Brain Heart Infusion agar containing five percent sucrose. The presumptive colonies were streaked on Congo red agar and incubated at 37°C. Formation of slimy and shining black colonies within 24 h of incubation was suggestive of exopolysaccharide production indirectly indicating their ability for biofilm formation.

Quantification of biofilm by Microtiter plate assay

Quantification of biofilm formation of the isolate was evaluated by microtiter plate assay (Stepanavoic *et al.,* 2000). M17 broth (230µL) was transferred to sterilised 96 well microtiter plates. To each well 20µL of activated culture was added and mixed thoroughly. Wells without added culture act as negative control. The plates were incubated aerobically for 24h at 37°C. The content of the plate was then poured off and the wells were washed three times with 300µL of sterile distilled water. The remnants after washing were fixed using 250µL of methanol per well for 15 minutes. Microplates was then emptied and air dried. After drying wells were stained with 250µL of Crystal violet (2%) for five minutes. Excess stain was rinsed off carefully by placing the microplate gently under running tap water, taking care not to remove the adhered matter. Microtiter plates were again air dried. The dye bound to the adherent cells was solubilized with 250µL of 33% (v/v) glacial acetic acid. The optical density (OD) of each well was measured at 570 nm using an automated microtiter plate reader (Robonik, India Pvt. Ltd). Based on the OD produced by bacterial films, strains were classified into the following categories: non biofilm formers, weak biofilm formers, moderate biofilm formers or strong biofilm formers. The results were expressed as the mean of three replications.

Strain was classified as follows :

OD ≤ ODc = no biofilm former
ODc< OD ≤ (2 ODc) = weak biofilm former
(2ODc) < OD ≤ (4ODc) = moderate biofilm former
(4ODc) < OD = strong biofilm former.
Where OD_c is the Optical density of the negative control

Safety assessment

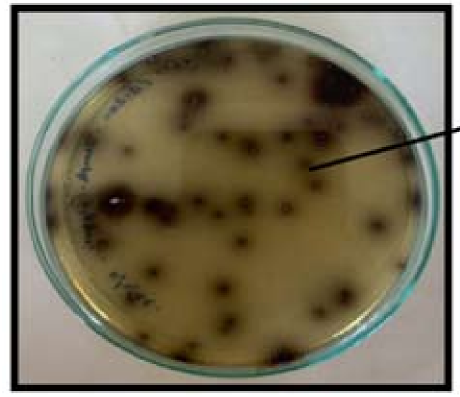
Safety evaluation of isolate was done by investigating their haemolytic activity and gelatin liquefaction potential. The presence of clear zones around the colonies in blood agar plates indicates beta haemolysis and green-hued colonies indicate alpha haemolysis and those which did not produce any zone was interpreted as gamma haemolytic (Barbosa *et al.,* 2010). For the detection of gelatinase activity isolates was streaked on gelatin agar slants and incubated at 37°C for 24 h. Liquefaction of gelatin was checked after 3h of refrigeration (Sahu *et al.,* 2019)

Results and discussion

Isolation and identification of Enterococcus sp.

Black hallow colonies in Bile esculin agar were considered presumptive for enterococci. Hydrolysing esculin in the presence of bile results in the formation of esculetin, which in turn reacts with ferric ions resulting in blackening of media (Chingwaru *et al.,* 2003). Microscopic examination of colonies revealed the isolates as Gram-positive small cocci arranged in pairs and short chains. The isolate was found to be catalase negative and oxidase negative.

The ability of the isolate to utilise different carbon sources was assessed using Andrade peptone water. The sugar utilisation



Colonies with black hallow precipitate indicating esculin hydrolysis

Fig 1: Colonies in Bile esculin agar

pattern of the isolates is given in **Table 1**. A change in the colour of media from pale tan to pink-red after incubation indicated a positive test for sugar utilization. The isolate was found to be a fast fermenter capable of utilizing Fructose, Galactose, Maltose, Lactose, Trehalose, Melibiose Sucrose, Cellobiose, Dextrose, and Arabinose within 24h of incubation without gas production. The potential to metabolise a wide array of carbohydrates enables enterococci to thrive in a diverse niche (Ramsey *et al.,* 2014). *Enterococci* are generally Voges Proskauer test (VP) positive (Facklam and Collins, 1989). In this study isolate was found to be positive for the Voges Proskauer test, indicating their ability to produce acetyl methyl carbinol. Enterococcal isolate obtained in this study possessed the ability to grow at 6.5% NaCl, pH 9.6, 10°C and 45°C, these specific physiological characteristics are recognized criteria to identify *Enterococcus sp.* to genus level (Manero and Blanch, 1999). Ability to multiply at 10^{0}C was limited and increase in turbidity was evident only after 72h of incubation. On checking the motility, growth was observed only along the stab line indicating their non-motile nature. Vincent *et al.,* (1991) identified *Enterococcus gallinarum* and *Enterococcus casseliflavus* as motile species of *Enterococcus.*

Isolate was identified at the molecular level as *Enterococcus faecium* DMAI03 with 98.35% similarity to *Enterococcus faecium* TEM1, an isolate from human infant faeces and was deposited in National Centre for Biotechnology Information (NCBI) with accession number OR105180.

Table 1: Sugar utilisation test

Fructose	+
Galactose	+
Inositol	-
Lactose	+
Maltose	+
Mannitol	-
Melibiose	+
Sucrose	+
Trehalose	+
Xylose	-
Cellobiose	+
Arabinose	+
Dextrose	+
Inulin	-
Ducitol	-

*(+) positive reaction (-) negative reaction

Antibiogram of the isolate by Disk-diffusion assay

Resistant pattern of *Enterococcus faecium* DMAI03 is given in Table 2**.** The result was interpreted as per CLSI guidelines (2020). The isolate was found resistant to four classes of antibiotics: Beta lactam, Glycopeptide, Tetracycline and Macrolide and hence adjudged as Multiple Drug Resistant (Magiorakos *et al.,* 2011). The isolate was found to be resistant to Vancomycin. Vancomycin resistant Enterococci (VRE) is identified as serious threat by Centre

Fig 2: Antibiotic susceptibility pattern of *Enterococcus faecium* in Muller Hinton Agar

Table 2: Antibiogram of the isolate using Disk diffusion assay

Antibiotics (Concentration)	Class of antibiotics	Zone of inhibition in mm	Result
Penicillin (10 units)	Beta lactam	Nil	R
Ampicillin (10 mcg)	Beta lactam	Nil	R
Linezolid (30 mcg)	Oxazolidinones	31.33±0.58	S
Vancomycin (30mcg)	Glycopeptide	Nil	R
Tetracycline X(30 mcg)	Tetracycline	Nil	R
Chloramphenicol (30 mcg)	Chloramphenicol	23.33±1.15	S
Erythromycin (15 mcg)	Macrolides	Nil	R
Rifampicin (30 mcg)	Rifamycin	21.33±1.15	S

*R- Resistant, S- Susceptible, I- Intermediate Results were interpreted as per CLSI guidelines, 2020

Table 3: Minimum inhibitory concentration of antibiotics against *Enterococcus faecium*

Antibiotics (Concentration)	MIC Break points for *Enterococcus sp.* (mcg/ml) established by CLSI	MICs of *Enterococcus faecium DMAI02*	Result
Penicillin (0.002-32 mcg/ml)	16	≥32	R
Ampicillin (0.016-256 mcg/ml)	16	≥16	R
Linezolid (0.016-256 mcg/ml)	8	1	S
Vancomycin (0.016-256 mcg/ml)	32	≥256	R
Tetracycline (0.016-256 mcg/ml)	16	≥256	R
Erythromycin (0.016-256 mcg/ml)	8	≥256	R
Chloramphenicol (0.016-256 mcg/ml)	32	1	S

*R- Resistant, S- Susceptible, MIC- Minimum inhibitory Concentration Results were interpreted as per CLSI guidelines, 2020

for Disease Control and Prevention. Isolate was found to be sensitive to Linezolid, Rifampicin and Chloramphenicol. High sensitivity to Linezolid in *Enterococcus sp.* has been reported by Arabestani *et al.* (2016).

The multiple Antibiotic Resistance index of *Enterococcus faecium* in the study was found to be 0.625. MAR index greater than 0.2 indicates high-risk (Krumperman, 1983). The higher the MAR index, greater the risk. Occurrence of starter bacteria with MAR index greater than 0.2 implies that household *thayir* could be a high risk source for Multiple Drug Resistant bacteria. Chandran, (2022) have reported the prevalence of enterococci in household samples of *thayir.*

Minimum inhibitory concentration (MIC) of the isolate

Minimum inhibitory concentration is defined as the minimum concentration of antibiotics for inhibiting the growth of the bacteria. Minimum inhibitory values greater than break point values given in CLSI guidelines were observed for Penicillin, Ampicillin, Vancomycin, Tetracycline and Erythromycin (Table 3) for this isolate. High MIC values against these antibiotics were observed for clinical enterococcal isolate by Abdulla and Abdulla, (2006). MIC values comparable with that of clinical isolates imply a potential public health risk.

Biofilm forming potential

Congo red agar assay was used to qualitatively evaluate the biofilm forming ability of the isolate. In this assay, Congo red reacts with beta glucans in exopolysaccharide producing black colonies which helps in distinguishing EPS producer from non EPS producer (Tsveteslava *et al.,* 2017). Colony characteristics of the isolate on Congo-red agar is given in Fig 4. The isolate appeared as black crystalline colonies on Congo-red agar. EPS production is indirectly linked with biofilm formation, however, Hou *et*

Fig 3: Minimum inhibitory concentration of Penicillin against *Enterococcus faecium*

al. (2012) reported that a positive result in Congo-red assay does not always implies biofilm formation. Quantification of biofilm formation was evaluated by microtiter assay. The microtiter plate reading of the isolate was found to be 0.93±0.52 and that of control was 0.7433±0.15. As per the guidelines, the isolate in this study was graded as weak biofilm former. Microbial cells within biofilms have shown 10-1000 times more antibiotic resistance than planktonic cells (Mah, 2012). In this study, the isolates were found to be weak biofilm former with remarkable resistance properties.

Safety assessment

Haemolysis and gelatin liquefaction are two properties that are linked to pathogenicity. Haemolysis indicates the ability of isolates to lyse Red Blood Corpuscles (RBC). *Enterococcus faecium* produced green hued colonies in blood agar indicating their alpha haemolytic nature. Gelatinase is an extracellular zinc metallo-endopeptidase secreted by *Enterococcus sp.* which can hydrolyse gelatin, casein, haemoglobin and other bioactive peptides (Koch *et al.,* 2004). Even though gelatinase is a common virulence factor in enterococci, the isolate in the present study, enterococci did not liquefy gelatin.

Conclusion

Enterococcus faecium isolated from household *thayir* samples were resistant to more than three classes of antibiotics. The prevalence of Multiple Drug Resistant enterococci in *thayir* is a real safety issue as such products can lead to the widespread of resistance genes. The findings pointed out that Multiple Drug Resistant enterococci can act as a concealed threat in *dahi/thayir,* the fermented dietary staple of Kerala. The observations endorse the possibility of household *thayir* samples being a potential reservoir for the dissemination of antibiotic resistance to human gut microflora.

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Conflict of interest

The authors declare that they have no conflict of interest.

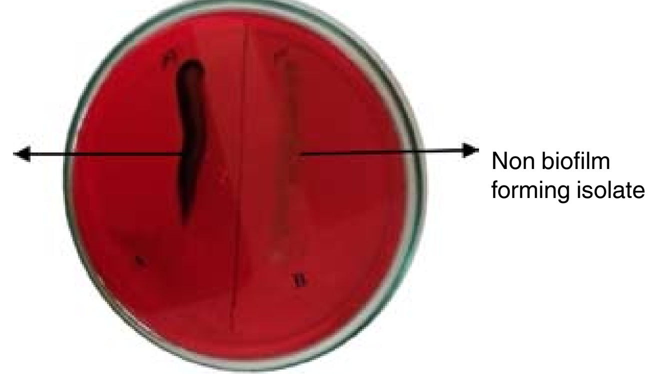

Fig 4: Colony characteristics in Congo-red agar,

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